Lipoids and Blood Platelets

WITH REFERENCE TO BLOOD COAGULATION

AND THE HEMORRHAGIC DISEASES

By

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Im Tatsachlichen grosster Pragnanz

im Hypothetischen ausserste Vorsicht (Wohlisch 1943)

When theory fancy free
Bids hither yon s the way
Calm fact exhorteth thee
Consider tyro' Stay (Orig)

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EDITORIAL NOTE

The author adopts the American spelling in the text

Bibliography:

(1) References are given in the text by numerals in square brackets corresponding to alphabetized bibliography in Appendix III

(2) Titles of journals are abbreviated in accordance with the widely used List of periodicals abstracted by Chemical Abstracts American Chemical Society (Columbus Ohio) 1951

(3) Abstracts (abstr) and 'personal communications' are indicated

(4) Authors are listed alphabetically In case of multiple authorship the second and subsequent authors' names follow in alphabetical sequence

(5) Diphthongs are replaced by separate letters Modified letters (accent etc) when not anglicized (e g ue German u) in the orig inal reference appear in the usual alphabet order but include the modification

(6) Hyphenated and compound names are listed in alphabetical order *under initial letter of surname including De Le Mac Mc Van* Von

Author

ABSTRACT

By specifically analyzing for the various active principles of plasma platelets tissues and their fractions much new information has been ob tained concerning the role of lipids and platelets in blood coagulation and in the hemostatic mechanisms in health and disease Analyzed compon ents are studied in artificial clotting systems especially a two stage *thrombin forming system Some 86 cases of bleeding disorders 32 new* born normal infants and their mothers and many normal adult bloods have been analyzed with respect to components of the clotting and hemo static functions

The detailed considerations embodied in the dissertation are encom passed under the following heads:

1) the importance of certain lipids especially cephalin

2) the normal need for platelets in plasma clotting

3) the particular significance of a platelet component which has many analogies to cephalin in the thromboplastic system

4) potentiation of the thromboplastic actions of cephalin of platelets and of tissue thromboplastin (to some extent) by a variety of experimental additives Part of this may be explained as a 'thromboplastin generation' through co participation of certain plasmatic components (antihemophilic globulin PTC etc) Part however may be the result of certain proteo lytic enzymes particularly trypsin 'disaggregating lipoproteins and thus rendering their phospholipid (and sometimes calcium) available for par *ticipation in the clotting reactions*

5) possible Ca containing and lipid containing 'intermediates in the thrombin forming reactions

6) myelin figure formation as an explanation of 'alterations of plate lets and certain other formed elements such as thrombocytes megakary ocytes and stromatolytic erythrocytes

7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanisms

8) the occurrence of many clinical disorders due to deficiency of plate let functions Thrombocytopenias denote deficient numbers (counts and total bulk in body) Thrombocytopathias are deficiencies of specific platelet components e g thromboplastic factor accelerator vasocon strictor (5 hydroxytryptamine) or retractor factor Such deficiencies can be clinically significant even when the platelet count is normal Bleeding in leukemias uremias etc may often be accounted for in these terms

9) the nature and modes of action of heparin and other 'antithrombo plastic' inhibitors *and of some antiproteases in relation to the mechan* isms discussed

10) the 'cephalin availability theory of the author as a useful working hypothesis to explain the importance of the natural thromboplastic phos pholipid Lipid release from platelet tissue or possibly plasma sources may very well be the long obscure 'trigger mechanism which initiates blood coagulation

11) The supplement reports 11 new experiments in the period 1956-1959 The versatile two stage method has been modified for the quantitative *study of each individual factor in the blood-clotting system* and adds some

new data concerning thromboplastic mechanisms These data contribute further support for the main thesis It is suggested that the term 'prothromboplastic' be used to designate the phosphatide (probably cephalin) which in conjunction with calcium ions and a diverse (experimentally) group of accessory factors constitutes the mechanism for activation of prothrombin to thrombin 'Availability of the lipid as a trigger mechanism' and its dynamic role in these activator mechanisms seem best explained in terms of a 'phospholipid transfer' hypothesis

CONTENTS

PART II EXPERIMENTAL

Lipoids and Blood Platelets

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AND THE HEMORRHAGIC DISEASES

PART I

BACKGROUND

FOREWORD

In presenting a dissertation embodying experimental work con ducted over a 27 year period (1929 1956) it is necessary to review the knowledge of the field at the commencement of this period and its subsequent progress to the present time in order to evaluate each contribution in the light of contemporary knowledge and theory Several volumes and possibly several authors would be required to review critically and in detail all the extensive literature of the blood coagulation field From the physiological approach alone several thousand titles are referred to in the monumental reviews of P Mora witz [346 347] and E Wöhlisch [505 506] and in more recent articles including those appearing in the Annual Reviews of Physiology two of them by the present author [133 140] The purposes of this disserta tion may best be served by a selection of those ideas and trends of thought which afford the background for the topics presented

A dissertation more than a definitive scientific publication re flects the interplay of ideas that influence the mind of its author and it is not inappropriate that it indicate to some extent the personal element in research In the period covered Science itself has under gone a change which is deserving of mention This change was grad ual in the beginning but it has accelerated in the last few decades of the twentieth century Characteristic of earlier periods was the emi nence of individual scientists whose discoveries opened up new fields or special areas within those fields often single handed Students and young investigators came to work in the laboratories of the dis tinguished authority and thus developed the various schools 'justly adding to the reputation and prestige of the master ' A fine example in our field was the Dorpat school under the leadership of Alexander Schmidt (1860 1895)

The modern scientific era has seen much larger numbers of sci entists working simultaneously in the same field Through scientific gatherings of regional national and international scope workers of kindred interest have frequent opportunities for exchange of ideas Thus all are kept fully informed of many lines of progress and of the definition of numerous problems in advance of their solution A not infrequent consequence is that the same solution (or almost) is reached simultaneously by several independent groups of workers perhaps widely scattered over the world An illustration of this may be cited from a field peripheral to that of the present inquiry In 1939 the Journal of the American Chemical Society simultaneously published four papers from different laboratories all of which must share the credit for the definitive solution to the problem of the chemistry of vitamin(s) K Much the same sort of thing is true of the recent

mention These established the clotting phenomenon as essentially a physiological property of the 'coagulable lymph' (Butt 1760 cf. 'coagulated lymph' of de Senac 1749) which we nowadays call plasma Malpighi (1666) and Ruysch (1707) had already noted the 'fibrous material obtainable from blood clots and clinicians with wide experience of ubiquitous blood letting talked of the 'cruor' or crassamentum' appearing after separation of the 'serosity' or serum Much was made of the 'buffing' of blood in disease This we now interpret as an increased erythrocyte sedimentation rate with the consequent easier observation of clotting sequences starting in the supernatant plasma rich layer The term fibrin which we owe to the French encyclopedists Chaptal [72] and de Fourcroy [99] was not really an advance since they used the crudest methods of preparation of the clot material which they tried to characterize by appearance and elementary analysis Mulder introduced the word protein in 1839 but it has been a long and difficult road still with unexplored bypaths to define individual proteins and among them fibrin and fibrinogen The latter term we owe to Virchow [482]

The 1830 1859 publications of P S Denis are of historical interest Denis' [102] most significant contribution all things considered was the practical preparation of his plasmine ' This was a precipitate obtained from plasma with strong solutions of sodium chloride and other neutral salts Redissolved on addition of water it subsequently underwent coagulation indistinguishable from the appearance of natural fibrin clot

The false impression that fibrin was the result of some 'spontaneous' change in a soluble percursor (fibrinogen) was first questioned by Buchanan [68 69] particularly in his observation that the washings from a blood clot could coagulate certain hydrocele fluids He compared this with the rennet curdling of milk and also did much to try and prove a cellular origin of the blood coagulant Buchanan's work was neglected until republished by Gamgee [183] in support of his confirmatory experiments Gamgee s method of preparing thrombin (as it has come to be termed) from blood clots was further improved by W H Howell [229 232] at the beginning of our current era

2 THE DORPAT SCHOOL

Alexander Schmidt of Dorpat established his famous school in the latter half of the 19th century From the master and his pupils came a large body of work and publications reviewed in Schmidt's two monographs [420 421] in 1892 1895 It is easy from our advanced modern viewpoint to be extremely critical of the crudity of their experimentation and highly theoretical presentations of their ideas It should be remembered however that the difficult biochemical problems of the coagulation field humble us even to this day Then again the 19th century discoveries especially in the field of ferments (or enzymes) aroused such interest that contemporary enthusiasm sometimes got out of bounds Analogies were suggested and their implications tacitly accepted with little of the caution demanded of more recent experimental science Schmidt used a crude technique of storing blood serum under alcohol to obtain his 'fibrin ferment ' which was a very weak

discoveries of several new factors in the blood clotting mechanism Here another consequence is manifest namely some uncertainty be cause of the difficulty in exact identification of the factors in question thus leading to a confusion in nomenclature Such confusion is more troublesome to the casually interested scientist than to those actually working on these problems however

In the coagulation field at least in America perhaps the last of the old school authorities was W H Howell of the Johns Hopkins School of Medicine in Baltimore This eminent scientist and distin guished teacher was quietly continuing his critical experimentation in a modest laboratory which he occupied as Emeritus Professor of Physiology during the period of the 1930's when the present author had the privilege of paying him numerous visits His friendly charm advice and critical discussions and warm personal interest and en couragement were of inestimable value in guiding a new researcher into the complexities of the blood clotting field

On the occasion of the annual meeting of the Federation of Amer ican Societies for Experimental Biology at Detroit in 1935 a small group of interested coagulationists met together and agreed to main- tain an exchange of ideas By common consensus this 'coagulationist' group has never become formally organized It has however con- tinued to meet at each annual meeting of the Federation and the sus tained interest has expanded with each succeeding year so that our mailing list now has over two hundred names There are similar groups of workers in other specialized fields and a very important result of their coordinated interests has been to secure inter society sessions and symposia at such gatherings as the Federation and the International Physiological and Hematological Societies' meetings Other significant contributions to the cross fertilization of ideas among specialized scientific groups are the numerous Research Con- ferences which are a distinctive feature of the modern scientific scene Some examples in which American coagulationists get to- gether are those promoted by the National Research Council the U S Public Health Service and the Josiah Macy Jr Foundation to mention just a few

With this broad perspective we may selectively summarize the early history of blood coagulation in what we may call the personal era and then proceed to a brief review of its modern status in which due credit for individual investigators need not be denied by present ing this largely from the depersonalized point of view

The topic of the blood platelets will be separately reviewed

CHANGING CONCEPTS IN THE CLOTTING FIELD

A HISTORICAL HIGHLIGHTS

1 EARLY ORIGINS: HEWSON DENIS BUCHANAN

The earliest significant efforts toward an understanding of the coagulation of the blood were made in the 18th century and the *Experimental Inquiries* of young William Hewson [218] associate of the famous brothers John and William Hunter are particularly worthy of

3 THE WORK OF HAMMARSTEN AND WOOLDRIDGE

Olof Hammarsten [205] in 1883 seemed to simplify Schmidt's theories by suggesting that 'fibrinoplastic substance' ('fibrino globulin') was not a component of the coagulant but appeared as a split product of fibrinogen when acted upon by the fibrin ferment However this eminent biochemist went on to considerable success in the purification of both fibrinogen and thrombin We owe to him the application of the 'salting out' technique to precipitate fibrinogen from plasma using ½ sat NaCl or ¼ sat $(NH_4)_2SO_4$ Weyl Heynsius and others (ref [371]) were then developing the use of neutral salts rather than the earlier acids and alkalies etc for the separation of all kinds of proteins The distinction between albumins and globulins on the basis of solubilities in such salt solutions was made in this era When oxalate or citrate (see later) replaced the older $MgSO_4$ etc as anticoagulants used in separating (centrifugally) the plasma from the blood corpuscles the preparation of fibrinogen and other clotting fac tors by the salting out method was much improved The technique of elementary organic analysis by combustion methods dating back to Liebig (1840) was continued and despite the relative crudity of the materials analyzed did give data on N S P and minerals e g Ca which contributed some information of real value in these early days of protein and other organic chemistry Hammarsten eventually pur ified fibrinogen until he was able to obtain up to 94 percent of its pro tein in the fibrin clot which resulted from treating its solutions with thrombin His thrombin was fractionated from fresh horse serum The conclusion from these careful experiments was that the small quantity of non coagulable protein unaccounted for was merely a minor contamination with what we would now term serum globulins Hammarsten's work on the calcium factor will be reviewed on p 8

L C Wooldridge [508] had considerable justification for some of his claims to priority over the Dorpat school in the investigation of phospholipids ('lecithins') in the blood clotting system He obtained many interesting experimental facts but his tissue fibrinogen theory and many aspects of his work were too unorthodox to gain him wide recognition Even the publication of his obscure works by Horsely and Starling in 1893 failed to shake the widespread acceptance of the thrombin theory

4 THE CALCIUM FACTOR Early work of Arthus Pekelharing Hammarsten Sabbatini

Alexander Schmidt was never convinced of the need for calcium in the thrombin forming reaction There had been previous suggestions [201 404] for a role of calcium in blood clotting but the significant evidence was provided by Arthus and Pages in 1890 [19] They used oxalates or fluorides to prevent clotting and restored it by simple re addition of calcium salt

Pekelharing [372 373] confirmed this and introduced citrates for the same purpose This worker improved upon Schmidt s methods for obtaining prothrombin Dilution and acidification of plasma with weak

and impure thrombin by modern standards His numerous experiments endeavored to search out all possible factors influencing the clotting mechanism Proplastic' i e fibrinogen containing liquids such as hydrocele fluid or plasma obtained from blood kept fluid with $MgSO_4$ and other neutral salts (cf Hewson [218]) were clotted by Schmidt's fibrin ferment He obtained good evidence for a precursor of the coagulant The terms thrombin (for fibrin ferment) and prothrombin (for its precursor) were introduced in his later writings Thus Schmidt has deservedly been called the father of the thrombin theory Many details of his particular theories and terminologies have long since been discarded and need not be reviewed A few words may be said however about some of the experimental facts In attempting to unravel the thrombin problem Schmidt did much to implicate (he thought) both (1) a plasma protein 'paraglobulin ' and (2) tissue or cellular factors which he called 'zymoplastic substances ' As to the first it did seem rational at the time to look for a proteolytic 'split product' accompanying the postulated enzymatic breakdown of fibrinogen to fibrin

Schmidt and his pupils did a great deal with the tissue materials noted under the second head namely zymoplastic substances ' These were obtained both in aqueous extracts and by means of fat solvents hence chiefly lipoidal in character Heat stability and many other properties were investigated The most significant conclusion about these zymoplastic agents was that they were not thrombin but could participate in the activation of prothrombin

The essential technique whereby Schmidt sought to characterize tissue fractions was first to obtain an alcoholic extract ('zymoplastic substance') then a succeeding watery extract (cytoglobulin') and its acetic acid precipitable fraction ('preglobulin') and finally an insoluble residue ('cytin') Of historical interest were the observations (1) zymoplastic substance promoted clotting under a variety of test circumstances some of which led to the conclusion (above) that it could participate in the activation of prothrombin to thrombin (2) the defatted cellular extractives especially preglobulin had considerable clot inhibiting powers e g for mixtures of $MgSO_4$ plasma and fibrin ferment (thrombin) (3) zymoplastic substance however was able to restore clotting in these inhibited mixtures

Schmidt s final summing up of his views on coagulation [421] included the idea of something normally present in blood which could be regarded as an antithrombin This term was actually introduced by Fano (1881) to designate the thermolabile (on boiling) agent which caused incoagulability of blood after peptone injections in the dog as first shown in Carl Ludwig s laboratory by Schmidt Mulheim (1880) Extracts from the medicinal leech contain a substance hirudin which Haykraft [217] showed to have similar 'antithrombic' powers

It was generally known in this era that (a) the 'thrombic action of fresh serum progressively decreased on aging even for a few hours (b) thrombin clotted plasma less readily than a fibrinogen solution (c) the weak thrombin preparations of that time had little if any capacity to produce coagulation (thrombosis) on intravenous injection into experimental animals

Even when the blood remained coagulable the clots would soon undergo lysis As little as 1/60 vol of normal dog plasma could prevent this indicating that normal plasma contains a powerful antifibrinolysin This however is reduced or absent in the above cases involving failure of liver function Nolf performed many noteworthy experiments but his theories were heterodox In brief he tried to bring into a dual clotting fibrinolytic scheme: (1) fibrinogen (2) 'thrombogen' which is possibly equivalent to the orthodox prothrombin although Nolf also identified it with the antifibrinolytic agent (3) 'thrombozyme' possibly equivalent to Schmidt's zymoplastic substance or Morawitz s thrombokinase since Nolf asserted that it was produced by certain white cells (thrombocytes and platelets) and by the endothelium of the capillaries Nolf accepted thrombin only as a by product of the reaction between the above three substances and not as the coagulant itself as in the thrombin theory He used the term 'thromboplastic' for a heterogeneous group of agents including wettable surfaces e g ground glass calcium oxalate in colloidal suspension chloroform and similar emulsification which assist in the colloidal reactions The thromboplastic agent intervenes solely to make the reaction possible between these (3 cited) precursors It acts as a catalysator And so also do the alcoholic or aqueous tissue extracts It may be remembered that Freund [174] and Bordet & Gengou [58] retarded blood and plasma clotting by use of paraffined surfaces

7 THE HOWELL THEORY

Around 1910 W H Howell and his pupils were re investigating (a) the thrombin fibrinogen reaction (Rettger 1910; Howell 1910) (b) the intravenous injection of thrombin (Davis 1910) and (c) the clotting of peptone and hirudin plasmas especially by glycerinized (Cecil 1911) tissue extracts In a 1911 paper Howell [230] carefully reconsidered the role of thromboplastic tissue extracts and because of their ability to clot the above mentioned inhibitor containing plasmas he concluded in favor of an alternative to Morawitz's theory namely that tissue extracts act only by neutralizing certain clot inhibitors Furthermore he argued for a type of (natural) inhibitor which does not merely antagonize active thrombin but actually prevents the formation of thrombin from prothrombin Hence the new term antiprothrombin' and the specific idea (Howell's 'thromboplastin') that tissue extracts particularly the thermostable lipoidal fraction act by neutralizing antiprothrombin

In 1912 independent of Zak [514] Howell [231] identified the thromboplastic agent as cephalin in some form of protein combination Lecithin whether from tissues or egg yolk was inactive Howell's cephalin preparation followed the method of Thudichum [464 465] pioneer in the field of brain chemistry Diakonow had separated 'lecithin from other brain lipoid fractions and shown that its hydrolytic products were glycerophosphoric acid choline and fatty acids such as oleic and margaric (ref [103]) Thudichum found that there were a variety of phosphorylated lipoids (phosphatides; phospholipids) differing especially in their nitrogenous basic groups Retaining the name lecithin for those containing choline the new term cephalin was given to

acetic acid separated this agent by a principle which we would now characterize as isoelectric precipitation Pekelharing however went to undue lengths to identify his material as a 'nucleo albumin' and suggested that it had a cellular perhaps platelet origin These were not useful ideas and they somewhat lessen the value of his contributions He did insist upon the importance of calcium salts in activating prothrombin to thrombin (fibrin ferment) but he loses us in his further speculations about its transfer of calcium to the fibrinogen in the act of clotting

Hammarsten [206] provided more convincing evidence of the specific importance of calcium in prothrombin activation while denying it any really significant role in fibrin formation By oxalation and 'isoelectric' precipitation of his fibrinogen and thrombin he obtained reagents which interacted to give a fibrin analyzing as little as 0 005 percent calcium This could reasonably be dismissed as a trace impurity (but see Howell's comments [238])

The question raised by citrates which are unlike oxalates in that they do not precipitate calcium was settled at the turn of the century by Sabbatini [417] who showed that they do remove Ca^{++} ions by forming inactive CaCit anion complexes

5 THE MORAWITZ-FULD THEORY

Morawitz [345 346] and Fuld & Spiro [181] clarified ideas concerning the role of tissue factors in the clotting mechanism Fuld's term 'cytozyme' was later adopted by a minority of workers notably including Bordet [55] whereas most European investigators accepted Morawitz's term 'thrombokinase ' The argument was still by analogy with a tacitly accepted enzyme hypothesis and it may be remembered that Pavlov (1899) had just discovered enterokinase to explain the intestinal activation of pancreatic trypsinogen into active trypsin The Morawitz Fuld theory did clearly state that both calcium ions and tissue factor were needed to convert prothrombin into thrombin

Morawitz [345] also gave us the term 'metathrombin' for the inactive form of thrombin in serum believed due to some combination with antithrombin which Morawitz following earlier leads by Alexander Schmidt thought he was able partly to reverse with an acid and alkali treatment

6 THE HETERODOXY OF NOLF

P Nolf [351] in the later 1900 s restudied the phenomena of peptone shock in experimental animals particularly in relation to the liver He not only confirmed earlier French workers (Contjean Gley Hedon and Delezenne) as to the hepatic origin of antithrombin (which Nolf preferred to call 'antithrombosin') but also gave good evidence that the liver produced fibrinogen and antifibrinolysin Nolf was especially interested in the fibrinolytic phenomenon pointing out the loss of fibrinogen due to appearance *of an active proteolytic enzyme (fibrinolysin)* in the blood partly explaining the incoagulability encountered not only in peptone shock but also in liver poisoning with phosphorus (Corin & Anclaux 1894) or with chloroform (Doyon 1905)

of prothrombin calcium and kinase (tissue extract) Howell's oppo site results' were thought to be due possibly to differences in the two prothrombin preparations or in the test substrate (purified fibrinogen vs dialyzed oxalated plasma) Howell failed to mention a much more significant point namely that Mellanby's 'kinase' was a crude aqueous tissue extract whereas Howell's 'thromboplastin' was purified cephalin Howell cites Fuchs' [177] suggestion that the prothrombin complex might be the same as the 'proserozyme' postulated by Bordet and Delange [56] No further comment is made in the review but I know from personal conversations with Howell that he was unimpressed with this idea Bordet's proserozyme was said to change to serozyme (prothrombin) on contact with wettable surfaces but heparinized plasma or prothrombin solutions are unaffected by the surface of the container Howell [238] did not bring the heparin cofactor into the antiprothrombic thesis but only into the antithrombic action of heparin Thus unlike hirudin heparin has no inhibitory effect upon the reaction between thrombin and fibrinogen although heparin added to plasma or serum causes the production in these liquids of a true antithrombin by a reaction with some unknown thermolabile constituent of the blood Let us anticipate some of the modern era of our theme to record the reasons for the refutation of the Howell theory This came about because of failure experimentally to verify the essential facts which the theory requires Here are the answers to some key questions:

1) Are significant amounts of heparin to be found in the blood normally? The best answer is that of Jaques [249] who used a reliable chemical assay method which failed to find more than 10 micrograms per 100 ml in normal human bloods This is only about 1/10 the amount of the heparin needed to keep blood from clotting in vitro Heparinemia can occur in peptone shock and other anaphylactoid reactions [255]

2) Can heparin form a prothrombin antithrombin type of complex? Because of its highly acidic groups heparin can bombine with the basic groups of many proteins etc (Fischer [162]) Chargaff [75] obtained evidence of a stoichiometric combination between heparin and protamine (a basic split product of salmon roe protein) It can form a polyanion complex with fibrinogen experimentally [42] No one however has isolated anything like the postulated heparin prothrombin compound The inhibitory actions of heparin have proved multiple and complex They are still insufficiently understood but much that is now known can explain these actions without need for postulating Howell's complex [425]

3) Does thromboplastin neutralize heparin and release prothrombin? As to the first part of this question let us for the moment identify thromboplastin with cephalin (thromboplastic phospholipid) Phosphorylated lipids are acidic in much the same way as the sulfonated heparins Hence a direct neutralization seems improbable chemically There is much more reason and evidence (e g Chargaff [75]) to think of both the phosphatide and the heparin as competing for the basic groups of proteins Cephalin combines stoichiometrically with protamine [74] Heparin the stronger acid can 'deviate' cephalin from this combination When heparin is made to react with the thromboplastic

those containing ethanolamine In 1915 Howell set his pupil Jay McLean to test in the blood clotting system all phosphatides described in the literature to that time An unexpected result will receive due consideration in a later paragraph The data for Howell's last paper [239] on The isolation of thromboplastin from lung tissue were completed just before his death in 1945 but it was his daughter Dr Janet Howell Clark who wrote it up for publication kindly submitting it for my appraisal before sending it to the publishers This paper showed the progress Howell had made toward characterizing thromboplastin as a protein compound of a phospholipid ' from which it was possible to remove the protein leaving a residue with marked thromboplastic activity the chemical nature of which has not been determined '

Howell [233] used a crude preparation of prothrombin simply obtained by precipitation of plasma with acetone washing with ether drying on a Buchner funnel and subsequently extracting with alkalinized water or saline None of his prothrombins failed to activate with calcium alone In fact many of them activated to thrombin 'spontaneously' and this was enhanced by treatment with $CHCl_3$ and other lipid solvents [71] Howell [229 232] also prepared a thrombin by 8% NaCl extraction of washed blood clots (see p 5) and could purify this by shaking with chloroform until it no longer gave any reaction to chemical tests for phosphorus These data caused Howell to reject the European thrombokinase idea and insist that calcium alone could activate prothrombin Reviewing these matters in his 1916 Harvey Lecture Howell [235] agreed that thermolabile (50° 60°C) aqueous tissue extracts or relatively thermostable cephalin do assist in prothrombin activation but he proposed an alternative explanation namely that under normal conditions the prothrombin is protected from the activating influence of the calcium ions by a combination of some kind with an inhibitory agent or antisubstance and that cephalin exerts its accelerating effect upon coagulation by neutralizing the influence of this inhibitory substance thus liberating the prothrombin so that calcium can convert it to thrombin This is the essential Howell theory

Howell's pupil McLean [326] in the above mentioned study of a variety of relatively crude phosphatides found some that instead of aiding had a marked inhibitory effect upon coagulation These included two preparations of 'cuorin' (method of Erlandsen 1907) and a 'heparphosphatide' (method of Baskoff 1908) In personal conversations with Dr Jay McLean during visits to Columbus Ohio around 1940 he told me how elated he had been to discover antiprothrombin But he had to leave for active service in World War I and missed the follow-up work in which Howell and Holt [242] and Howell [237] established the more important aspects of this anticoagulant action including need for a plasma co-factor and identified the agent not as phosphatide but as a sulfonated polysaccharide amine which Howell called heparin In his 1925 Pasteur Lecture [236] and 1935 Physiological Review article [238] Howell clearly identified his antiprothrombin with heparin He did note that Mellanby [330] was not able to obtain any evidence that heparin combines with prothrombin finding that it did not prevent the activation of prothrombin in a mixture

titative in many experiments Yet one finds all sorts of inconsistencies and contradictions For example compare (a) every fibrinogen solu tion which has been tested has coagulated with calcium chloride alone and the statement (b) but a consideration of the properties of kinase and prothrombin and the constitution of plasma point to the conclusion that calcium alone will not clot fibrinogen solutions As 'thrombo kinase' Mellanby used diluted testicular extracts from the bled cock erel after the manner of Rauschenbach (1881) With calcium this readily clotted his 'fibrinogen' and he properly interpreted this as ev idence for prothrombin contamination in the substrate In this early work [327] Mellanby did not separate prothrombin but merely deduced its presence and activation from crude experiments especially varying the concentration of kinase

Mellanby's 1909 thrombin (fibrin ferment) was simply the fluid expressed after complete coagulation of a fibrinogen solution by kinase and calcium chloride It was with such reagents that he at tempted to quantitate the thrombin fibrinogen reaction both as to clotting times and fibrin yields He varied (a) calcium and other alka line earth salts (b) neutral salt (NaCl) concentrations (c) acid (HCl) or alkali (NaOH) and (d) oxalate citrate fluoride etc

In 1930 Mellanby [328] presented a purification technique for 'pro thrombase ' which was his term for prothrombin based upon the pre vailing enzyme idea Starting with dilution and acidification patterned after the earlier methods of A Schmidt and Pekelharing and essentially anticipating what we would now call an effort toward isoelectric precip itation he triumphed over many difficulties Thus the precipitate obtained from the diluted plasma is a complex mixture The greater portion of it consists of prothrombase fibrinogen and serum globulin but the pigments of serum cholesterol and thrombokinase (I) are al ways present in variable quantities The separation of prothrombase from this complex precipitate has presented considerable difficulties Nevertheless by diluting with lime water and bubbling CO_2 through to pH 6 8 7 0 and calcium bicarbonate (thus formed) about 0 0092 per cent he claimed splitting of the prothrombase from the complex The amount of calcium was said to be too small to activate the prothrom base in the time required for filtration and subsequent precipitation by acetic acid to pH 5 3 The final product was dried with acetone (cf Howell see p 10) Admissions of its frequent contamination with thrombin conflict with assertions of no spontaneous activation e g on dialysis Mellanby's product did have considerable potency and in some tests was partly stable to boiling for 5 min at pH 7 8

Addition of tissue extract alone (thrombokinase) to a solution of prothrombase always causes the generation of a corresponding quan tity of thrombase in the course of a few hours This fact holds good even when the prothrombase has been prepared from solutions contain ing no calcium salts i e oxalate plasma and the replacement of cal cium bicarbonate by sodium bicarbonate in the second stage of the pro cess However minute amounts of calcium have a marked effect on the velocity of activation Hence Mellanby balked at any final conclusion thus " no definite statement on the place of calcium salts in the reaction may be made until thrombokinase has been isolated in a pure condition

lipoprotein from lungs [87] the phosphatides are split off the protein carrier and a heparin-protein compound results This compound in losing its thromboplastic property has acquired a new one it exerts a markedly anticoagulant effect [83] ' The protein moiety of the thromboplastic agent must obviously also be considered Furthermore the plasmatic cofactor (cf [242]) has been shown [67 151a] to inter vene in the antiprothrombic as well as the antithrombic actions of hep arin and this requires further explanation As to the second part of the question real evidence is still lacking that prothrombin was bound in the first place

4) Can prothrombin be activated by calcium salts alone? This essential corollary to the Howell theory is seriously questioned by more recent work including data presented in this dissertation in which it will appear from experiments with both Howell type and with newer types of prothrombins that cephalin or tissue thromboplastin play a direct role along with calcium in the conversion of prothrombin to thrombin Furthermore wholly new factors [363 364] have now be come recognized as equally essential in the highly complex thrombin forming reactions In summary then the Howell *theory* was too simple and has not stood up to the inexorable pressure of more modern experimental facts

8 J MELLANBY STUDENT AND LEADER IN THIS FIELD

In 1909 J Mellanby working in the Physiological Laboratory at Cambridge (England) as George Henry Lewes' student published two [327] noteworthy papers in the field of blood coagulation and in his maturity he returned to this field with further investigations in the 1930's [328 329]

Mellanby first employed fowl plasma as recommended by Delezenne (1897) to prepare fibrinogen by a dilution and acidification technique in principle similar to the techniques of Alexander Schmidt [420 421] He rejected Hammarsten's [205] salting out method because of baneful influences of salts on proteins and stated it is clear that if fibrinogen is a globulin the best method to prepare it is to dilute and neutralize any fluid containing it This procedure will certainly precipitate all the globulins contained in the fluid used but there has not yet been adduced a certain proof that any globulin other than fibrinogen exists in plasma As a matter of fact the majority of experimental results point to the conclusion that fibrinogen is the only globulin contained in plasma [327] This error is not only con founded completely by more modern knowledge (ref [114]) but was untenable even with the data on plasma and serum proteins available in Mellanby's time Furthermore *it is inconsistent with many observations in his own work* Elsewhere Mellanby cited Pekelharing [373] as identifying prothrombin with 'kinase ' We have noted (p 8) that this authority did believe prothrombin to be a 'nucleo albumin' of cellular origin but *he was very clear that it was a plasma component* and quite distinct from the zymoplastic cellular substance of Alexander Schmidt

Credit must be given to the attempts of the Cambridge ' student to further the *knowledge of blood clotting* including his efforts to be quan

1935 [381]) Many including Alexander [6] and ourselves [147] have come to prefer $BaSO_4$ In their 1938 purification of prothrombin by CO_2 (under pressure) elution from the $Mg(OH)_2$ plasma adsorbate and its subsequent transformation to thrombin by means of calcium and lung extract the Iowa workers were assisted by W H Seegers [435 428] After establishing his own laboratory at Wayne University Detroit Seegers and his colleagues further purified prothrombin [424 431 425] and thrombin [433] from bovine plasma to give us the most potent and possibly the purest preparations to date There still remain some questions of trace impurities and it must be remembered that both adsorbents and precipitants yield several other components of the clotting system the presence of which in the final product may call for some critical comment

B MODERN VIEWS ON BLOOD CLOTTING

The following selective outline of present day (1956) knowledge is presented with slight personal bias but is essentially the consensus of the modern 'group thinking ' All workers agree that many details and perhaps still undiscovered factors await further exploration and that the present 'working hypothesis ' useful as it is especially in many clinical applications yet fails to give an adequately detailed answer to the deceptively simple but truly involved questions: (1) why does blood not clot in the normal circulation and (2) why does it clot (a) in thrombotic conditions and (b) when shed? To unravel the complexities we shall try to analyze the clotting mechanism step by step bearing in mind however the inter relatedness and frequent simultaneity or overlap of the several and successive reactions

1 SKELETON SCHEME OF CLOTTING REACTION

Figure 1 presents an intentionally over simplified scheme It merely indicates the two 'classical' phases of clotting The essential and final (second) phase is the conversion of the plasma protein fibrin ogen into fibrin clot through the physiological intervention of thrombin From one point of view this reaction is colloidal namely a change from 'sol' to gel' involving many physico chemical considerations including surface electrical charges depending upon polar groups of the reacting molecules and the 'ionic atmosphere' of the surrounding salt rich medium From another point of view it is enzymatic refer ring to a specific mode of action of thrombin

Thrombin is not normally present in the circulating blood Indeed there are antithrombic factors naturally present to inhibit or remove any thrombin which might possibly form in the blood The plasma does contain a precursor protein prothrombin The classical first phase of clotting then is concerned with the conversion of prothrombin to ac tive thrombin This is particularly complex and will require a search ing analysis of what our scheme collectively calls 'activators ' Possi bilities of inhibition of the activator mechanisms are indicated in the scheme and will also require investigation It is questionable whether we can group such first phase inhibitors as 'antiprothrombic' without becoming involved in the old controversies of the Howell theory

Mellanby's 'thrombase' (thrombin) described in a later paper [329] was obtained by slightly modifying the prothrombase method so as to permit conditions favorable to 'spontaneous' activation

We have cited the foregoing in some detail in order to emphasize the unsatisfactory state of experimentation with the blood coagulation problem as late as the 1930's Fibrinogen had come a long way with Hammarsten [205] and Howell [230] but prothrombin and 'kinase' and even Howell's [232] purified thrombin were far short of critical requirements It is in fact all too evident that most workers preferred to avoid facing the issue of contaminant impurities Most deductions were made as if these did not exist even if at times this meant a con siderable twisting of the experimental results It would not seem that it was asking too much by 1930 to ensure that fibrinogen preparations were free from all traces of prothrombin Yet nowhere can we find a product which failed to clot with calcium and tissue extract except possibly in some of the earlier experiments of Howell [230] Most of the time however Howell only used calcium salt in the control test since according to his theory this is all that is needed to activate prothrombin

9 MILLS: PRACTICE VS PRECEPT

C A Mills of Cincinnati was the other notable authority whose personal interest and exchange of ideas played a role in the present writer's entry into the blood clotting field at that time Of his volumi nous writings [334 337] little will be said in this review We do recall however Mills' insistence on a critical control of fibrinogen prepara tions for freedom from prothrombin Mills used cephalin as well as calcium in the preliminary test Unfortunately much of Mills' magnif icent conceptual approaches ended in data which must be discredited because of the failure to realize in practice what he preached in theory The critical control was inadequate when most needed to support the interpretation of his experiments The fact was that Mills [338] ob tained clotting in his 'prothrombin free' fibrinogen on adding lung ex- tracts or platelets in the presence of calcium This led him back to the heterodoxy of a second type of clotting and even to the nomencla ture of Wooldridge's [508] 'tissue fibrinogen '

The refutation of Mills' experiments and conclusions was made in 1934 by the Iowa pathologists Smith Warner and Brinkhous [442] In searching for a suitable fibrinogen these careful workers finally adopted the technique of adsorption with $Mg(OH)_2$ for removal of traces of prothrombin as previously recommended by Fuchs [180] Their second contribution was in the preparation of the lung extract itself Being highly vascular lung is ordinarily full of blood the pro- thrombin from which can appear in crude aqueous extracts By per fusing the lung with water or saline the Iowa workers were able to ob tain a subsequent prothrombin free extract This did not clot the critically prepared fibrinogen even on adding calcium

Prothrombin adsorption techniques were not new Pickering [375] reviewed a variety of reagents which had been tried for this purpose We may list $Ca_3(PO_4)_2$ (Bordet & Delange 1914 [v 55]) $BaSO_4$ (Dale & Walpole 1916 [98]) $Mg(OH)_2$ (Fuchs 1929 [176]) $Al(OH)_3$ (Quick

uncertainties but have clearly proved their value in many exacting quantitative tests *Particularly significant are* data which have been obtained with <u>physical measurements</u> e g opacity rigidity and tensile strength of clots light scattering (differential refractometer) streaming double refraction diffusion viscosity electrophoresis ultracentri fugation and electron microscopy Solubility studies [89] are very valuable in preparative procedures when worked out in relation to the five variables: (1) temperature (2) pH (3) ionic strength (4) protein concentration and (5) content of water miscible organic solvent e g ethanol zinc glycine etc Monumental contributions in these areas of our field have come from the late E J Cohn's Department of Physical Chemistry and Plasma Fractionation Laboratories at Harvard [114]

a) <u>Fibrin yields</u> The modern chemist seeks to follow the course and kinetics of reactions especially enzyme actions by timing changes in the substrate as well as by measuring amounts of the final products This logical approach is beset with technical difficulties in the case of the thrombin fibrinogen reaction The extent to which these may be overcome is illustrated in the *selected references* [159 348 60] Fibrin yields as measured by the amount of coagulated protein recoverable in washed clots is of course primarily dependent upon the initial amount of fibrinogen available That the thrombin concentration determines only the <u>rate</u> of the clotting reaction but not the ultimate fibrin <u>yield</u> is clearly shown in Figures 3 and 4 These are from experiments with purified fibrinogen and thrombin in the author s laboratory [60] Figure 3 shows effects of varying the relative thrombin concentration a thousandfold but employing a constant amount of fibrinogen Despite clotting time (see below) variations between 18 sec and 30 min the final (10 day) fibrin yields are identical and all 100 percent of the original (coagulable) protein in the fibrinogen These findings are strong evidence for the enzymatic nature of the thrombic action Figure 4 shows fibrin yields with time employing four different thrombin concentrations The linearity of the logarithmic plots (of residual substrate concentrations) within small limits of experimental error indicates a first order reaction [273] again consistent with an enzymatic activity *of thrombin*

NOTE: The results do not rule out a pseudo first order reaction in which preliminary phases might be slower than the action of thrombin Other things which do <u>not</u> affect the fibrin yield within reasonable limits are (1) temperature (2) pH (3) salt concentration e g NaCl $CaCl_2$ (4) 'fibrinoplastic'* colloids e g acacia (except for minor occlusion effects) (5) minor partial denaturation of fibrinogen (profibrin of Apitz 1937) Things which <u>may</u> affect fibrin yield include (a) extremely low fibrinogen concentrations (0 002 0 003 percent) where 'soluble fibrin ' i e intermediate polymers of insufficient degree of aggregation forms a significant proportion of the end product according

*Ferguson (1940 [134]) proposed a revival of this old term of Alexander Schmidt in order to indicate agents which shorten clotting times through nonspecific effects best interpreted as adsorptive or similar ways of bringing fibrinogen and thrombin reactive groups' together and thus facilitating their interaction

2 EXTENDED SCHEME OF CLOTTING AND HEMOSTATIC MECHANISMS

Figure 2 presents a more detailed scheme of the major factors in blood clotting and hemostasis with some indication of interrelation ships For clarity all inhibitor factors are omitted and must be con sidered separately Briefly summarized the following ideas are covered

1) Hemostasis is a physiological function involving:
   (a) vascular integrity and vasoconstrictor mechanisms
   (b) platelets (and other formed elements) participating in the cell thrombus
   (c) fibrin clot
2) Vascular factors will not be considered except to mention:
   (a) vasoconstrictor role of serum serotonin derived (in part) from 5 hydroxy tryptamine of platelets
   (b) role of damage to vascular endothelium in (i) initiating plate let participation (adherence clumping breakdown) and (ii) af fording site for fibrin deposition
3) Platelet factors (incompletely reviewed) including:
   (a) the above vasoconstrictor (2a)
   (b) a platelet component which reacts with plasma factors in 'thromboplastin generation '
   (c) a platelet 'accelerator '
   (d) platelet retractor factor
   (e) a role of thrombin in the platelet mechanisms
4) Plasmatic thromboplastic factors (AHF PTC etc) are indicated as reacting with platelets (3b) in 'thromboplastin generation '
5) Injured tissue sources of a 'complete' thromboplastin are indicated
6) Prothrombin is the inactive precursor of thrombin in plasma nor-mally activated by Ca^{++} and thromboplastin in the presence of (7) and (8) which are two essential plasma 'co factors '
7) Proconvertin yields convertin in the presence of Ca and thrombo plastin
8) Proaccelerin yields accelerin under the influence of a trace of thrombin
9) Active thrombin formed by the indicated reactions
10) Conversion of fibrinogen into fibrin 'Serum factor' (p 21) should be included
11) Clot retraction subsequently results from action of a platelet factor (3d)
12) Fibrinolysis and clot resolution result eventually from activation of the proteolytic enzyme system whose components are also in-dicated in the scheme
13) Various inhibitors should be included in the concepts outlined above (see pp 29 33)

3 *THE THROMBIN-FIBRINOGEN REACTION*

Most of the early work in this area was poorly quantitative and be set with uncertainties and errors caused by impurity of the reagents used Modern materials are by no means completely free from all

but show clots on subsequent thawing This may indicate that traces of thrombin can act on fibrinogen slowly even in the frozen state but it is just possible that the clotting occurs in the act of thawing

4) pH: There is an ill-defined pH optimum for the thrombin-fibrinogen reaction with some dependence upon the experimental con ditions It is important that the normal pH of the plasma (about 7 4) is close to this optimum Clotting times are prolonged but only slightly until we approach the extremes of (a) 5 3 in the acid range or (b) 10 in the alkaline region In (b) we run into the 'reversible in hibitor' problem (see (7)) of insufficient polymerization of the fibrin ogen [466] This may also be a factor in the acid region [405]

5) Ionic strength: The most important action of sodium chloride and most common neutral salts is a delay in clotting times with in creasing ionic strength [115] Numerous data on this go back all the way to William Hewson (1770) Polyvalent anions [191]e g ferro cyanides ferricyanides have especially marked inhibitory effects Table I summarizes an experiment of the author's [145] The exper iment shows that the inhibitory action of strong neutral salt (4 5% NaCl) which can be removed by subsequent dilution does not alter fibrinogen or thrombin and only prevents the appearance of visible fibrin in a mixture of these two agents Confirmation of the fact that clotting times become progressively shorter the longer the thrombin fibrinogen mixture is held with the 4 5% NaCl before dilution is re garded as evidence that the essential step in fibrin formation proceeds independently of the presence of the salt which merely inhibits or re-tards the gel formation (precipitation) cf Apitz [18]

6) Specific ion effects: Calcium is anomalous in that it definitely accelerates the clotting time of thrombin fibrinogen mixtures in a narrow range of low concentrations e g 0 008 0 032 molar in one of our experiments although this depends on total ionic strength [115 310] By removal of the Ca effect EDTA hinders the fibrinogen fibrin transition [409]

7) Reversible inhibitors: In an extensive recent study by Shulman [438] 42 (out of 80) substances tested markedly delayed or inhibited clotting in thrombin figrinogen mixtures under this author's test con ditions This type of inhibition is reversible by dialysis Important conclusions as to the 'polymerization process by which fibrinogen is transformed into fibrin can be drawn from these and other experi ments We shall consider this topic in a later paragraph

8) 'Fibrinoplastic' colloids [60]: The shortening of clotting times by these agents has been mentioned in the proceding section on fibrin yields and the footnote on p 17 suggests an explanation of their mode of action Gum acacia is of some importance in that it is used to stabilize the reactivity of fibrinogen in the Iowa two stage prothrom bin (and thrombin) bioassay [443] Protamine has similar effects [134]over a wide pH range We have also noted it with a highly puri fied lactoglobulin Also see 'platelets (p 51)

9) Partial denaturation of fibrinogen [60]: The present author s view is that the effect of this is due to the same general type of fibrin oplastic' action as noted under 8) That all of the denatured fibrinogen is included in the fibrin clot may be evidence of an adsorptive or oc clusion phenomenon The Wohlisch [504] denaturation theory' of clotting is now largely discredited

to Morrison [348]; (b) occluded proteins [348]; (c) reversible inhibitors of the type studied by J D Ferry S Shulman et al in the Wisconsin Laboratories [160 438 440] for the same reason; (d) fibrinolytic enzymes if active which are apt to be contaminants of thrombin and some fibrinogen preparations and can destroy fibrinogen and fibrin at approximately equal rates according to experiments in our laboratory [291]

b) Clotting times The earliest efforts to obtain quantitative information about the clotting process were by measurement of the time required to form a visible or a solid clot Because so many factors enter into the determination of any 'clotting time' the whole concept would seem to be fraught with extreme empiricism In fact it is possible to advance the view that there is no such thing as clotting time since the blood does not normally clot in the circulation so that we must merely be measuring the results of highly artificial conditions In actual practice however these conditions can be defined sufficiently to give clotting time determinations real value in following and interpreting the clotting phenomena As a technical point particularly referring to experimental systems of artificially isolated clotting agents most workers prefer to time the first appearance of a definitely visible clot (fibrin strands) rather than a solid gel (tube invertible) Both end points are empirical and depend upon a certain amount of fibrin formation by no means indicative of the ultimate fibrin yield The appearance of successive crops of clots when a weak thrombin is used is a well known phenomenon Factors known to influence clotting time include

1) Concentration of thrombin: the stronger the thrombin the shorter the clotting time other things being equal This is a common feature of enzyme reactions Figure 5 shows an experiment from the author's laboratories in which over at least a 10 fold range of relative thrombin concentrations the clotting times obeyed an 'inverse law' an old idea in the coagulation literature [163] It is perhaps unfortunately very difficult to define the precise experimental conditions for obtaining this result In most experiments the deviation from a linear relationship is significant It may still be possible however to translate clotting times into relative thrombin concentrations ('units' of activity) by use of this type of reference curve (simple plot) obtained under closely similar experimental conditions [20]

2) Concentration of fibrinogen over a considerable range this has remarkably little effect on the clotting time which is not so strange remembering that we time only a partial change in the substrate (see above) The quality of the clot varies however and may affect reading of the end point Particularly with very weak fibrinogens a poor clot and the probability of slower and less complete polymerization (referred to under fibrin yield) may cause longer clotting times

3) Temperature: It has long been known that cooling retards the reaction while warming accelerates it up to temperatures where complications appear because of thermal denaturation of the fibrinogen Thrombin is much more heat resistant but this depends on pH salt content and other considerations Some plasmas and impure fibrinogens may be completely clear when frozen and kept at 20°C

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10) Adsorptive removal of thrombin: Compared with whole blood or plasma clotting (see later) the surface of the container has little effect on the thrombin-fibrinogen reaction An exception is seen in the case of very weak thrombins where adsorption onto glass etc removes part of it from solution and hence lengthens the clotting-time This can be minimized by coating the tube with non-wettable silicone [60] It is a common experience that poorly washed glassware thus contaminated with a trace of thrombin can clot fibrinogen solution stored therein later

c) Nature of fibrin formation Progressing from the uncertain experiments of Apitz [18] Laki and his colleagues [278 274] produced evidence of a step wise process in the formation of fibrin from fibrinogen under the influence of thrombin The latest interpretation of these and other data was well brought out in the presentations and discussions at the 1951 Conference of the Josiah Macy Jr Foundation [2"4] J T Edsall (representing the Cohn laboratories) J D Ferry (formerly of the Cohn team but now at Wisconsin) K Laki (now at Bethesda Md N I H Laboratories) D F Waugh (Massachusetts Institute of Technology) were the chief authorites at this meeting with the present author contributing a minor role The modern concept of fibrin formation is as follows

Fibrinogen is a fibrillar protein whose long filamentous molecules approximate 38 A in diameter and 700 A in length (usually) with a molecular weight close to 500 000 (perhaps 330 000) In solution these large molecules moving with Brownian movement exhibit considerable swirling and 'steric hindrance ' During clotting however they line up 'like logs in a stream' and unite end to-end and also side to side until the 'polymerization aggregates' are large enough and hydrophobic enough to separate from the watery solution as visible fibrin Fibrin filaments or needles were seen to appear during clotting under the ordinary transilluminating microscope by Ranvier (1873) and Schimmelbusch (1885) Stübel in 1914 and Howell in the same year demonstrated them very clearly with the dark-field microscope Howell [234] discussed the problem of the fibrin gel formation in 1916 Electron microscopy first used for observing fibrin by Wolpers and Ruska [507] in 1939 has been used to support the modern views by Schmitt [422] by Hawn & Porter [212] Hall [204] Kaesberg & Shulman [264] and others Even the electron microscope does not resolve the fibrinogen molecules and the simplest polymers or protofibrils but it does show variations in meshwork density and the fibrillar composition together with cross striations of the fibrils which appear uniform under the ordinary (including dark field) microscope Consideration of clotting conditions (see above) in relation to electron microscopy and many physical measurements (see earlier) affords strong support for the modern concepts Thus under inhibitory conditions much smaller aggregates are formed and resemble the normal early or lag phase in which there is yet no visible clot but viscosity birefringence opacity and other data indicate some intermediate polymerization Under some conditions the fibrin polymer is reversible undergoing disaggregation and resolution e g in urea guanidine lithium bromide (strong solutions) etc [307] Irreversible and urea insoluble clots have recently been shown to depend on two things

namely (1) calcium [266] and (2) a special 'serum factor' by Laki & Lóránd [277] Lóránd [304 305 307] and Shulman [439] An earlier indication of this was found in 1944 by Robbins [405] in studying the somewhat similar problem of 'solution' of fibrin in acid (0 03 *M* HCl) or alkali (0 5 *M* Na_2CO_3) These data assist in explaining some of the peculiar actions of calcium when present in the thrombin fibrinogen (+ serum factor) mixture (see above) the 'serum factor' being a common contaminant of 'purified' fibrinogen (but see p 65)

It is suggested below how a limited attack on the fibrinogen mole cule activates polar groups which enable the altered substrate mole cules to aggregate in the polymerization processes The electrical charge distribution on fibrinogen molecules has been studied and there is good evidence that Coulomb forces play a role in the preliminary phases of aggregation Salt inhibitors (see ionic strength) are to be interpreted (Ferry [157]) as 'shielding' of the polar groups by the charged 'ionic atmosphere' contributed by the salt Further stages involve van der Waal forces (e g adsorption etc) and perhaps spe cific chemical bonds (currently debatable) [158] At all events the final steps do not involve thrombin Like other enzyme systems thrombin is not truly a part of the fibrin end product It is a practical fact however that a considerable amount of thrombin is adsorbed onto the fibrin clot [434] Indeed upon this depended the success of early methods of obtaining thrombin by extraction from washed blood clots

d) Thrombin This is by no means the simple proteolytic enzyme suggested by the 19th century coagulationists It is unlike trypsin [458] Most of the alleged evidence for its proteolytic powers [219 380] can be dismissed as due to contaminant fibrinolysin [149 462] or possibly as a certain degree of solubility of fibrin [247] However Sherry and his associates [437] have recently shown that fibrinolysin free thrombin can hydrolyze certain bonds in some synthetic polypep tides e g TAME (tosyl arginide methyl ester)

Lóránd [307] has contributed some provocative new ideas He uses Seeger's citrate thrombin (see p 23) and (a) bovine fibrinogen or (b) human fibrinogen [308] to show

1) non protein N is liberated pari passu with the clotting action of thrombin;
2) the glutamic acid missing from the fibrinogen after the action of thrombin can be identified in a 'fibrino peptide which has been isolated and characterized [309 306];
3) in bovine fibrinogen thrombin is thought to split arginine glycine bonds [30]; (NOTE: It does not do so in the case of insulin according to Middlebrook)
4) alanine replaces glutamic acid in the N terminal amino acid residues of human as compared with bovine fibrinogen but the general process is the same
5) electrophoretic studies show a characteristic pattern and the paper strip method permits characterization of the split products The pattern with thrombin is quite different from that produced by fibrinolysin

Lorand concludes that

a) the 'fibrino peptide' is a by product which does not partici pate in the fibrin polymerization but does account for the

10) Adsorptive removal of thrombin: Compared with whole blood or plasma clotting (see later) the surface of the container has little effect on the thrombin-fibrinogen reaction An exception is seen in the case of very weak thrombins where adsorption onto glass etc re moves part of it from solution and hence lengthens the clotting time This can be minimized by coating the tube with non wettable silicone [60] It is a common experience that poorly washed glassware thus contaminated with a trace of thrombin can clot fibrinogen solution stored therein later

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of prothrombin e g by calcium ions and thromboplastic (thrombokinase) factors with possibilities of inhibitors which have merely been confirmed with the modern 'purified' prothrombins Contamination with (a) traces of activators (b) traces of inhibitors (c) fibrinolytic enzyme (or precursor etc) (d) perhaps other factors may still need critical consideration even in the best modern preparations

b) Nature of prothrombin [425] Prothrombin is a specific protein accompanying the globulin fraction(s) of the plasma proteins Its quantity in the blood (15 mg/100 ml) [425] is very much less than that of fibrinogen (280 mg/100 ml) In fact unlike the latter it does not show an independent peak in plasma electrophoresis [491] Seegers and his colleagues have prepared the most potent hence presumably *the purest prothrombin to date and their work provides the most* reliable data on its characterization [425] Seegers believes that most of the difficulties in obtaining a biochemically pure prothrombin come from some activation and other marked alterations in the molecule during the purification storage and experimental procedures The purest preparation examined by Lamy & Waugh [280] was monodispersed in the ultracentrifuge but with some boundary spreading Its physical constants led to computation of the molecular weight at 62 700 and the shape of an ellipsoid molecule of 119 × 34 A Chemical analysis yields a trace of S (cystine and methionine) some 4 3 percent polysaccharide carbohydrate [431] and 3 75 percent ash Perhaps because of 'derivatives' (Seegers) prothrombin solutions show more than one electrophoretic component but the major component constitutes 70 90% of the total [432] The thrombin yield may depend upon changes during storage in the dry (lyophilized) state but generally averages 23 000 units* (two stage assay) per mg tyrosine or 1400 units/mg dry weight Highest: 32 300 35 800 *

c) Biophysics of thrombin formation [280]

(1) Biothrombin is a convenient term [280] for the thrombin obtained from purified prothrombin in the conventional manner i e addition of calcium salts tissue thromboplastin accelerator globulin (pro)convertin etc Maximal *transformation to thrombin is obtained* only when the prothrombin concentration is relatively low For physical studies concentrations of prothrombin of the order of 0 5 percent must be used (about 10 100 times as concentrated as used to obtain maximal activation) Under these conditions not more than 50% of the 'maximal' thrombin yield can be secured With the (lung) thromboplastin allegedly removed by high speed centrifugation subsequent ultracentrifugation shows a single symmetrical peak having an average sedimentation constant S ~ 4 8 corresponding to that of the original prothrombin Some boundary spreading suggests polydispersity however The specific activity of the material at the peak was 30 000 (thrombin) units/mg tyrosine

(2) 'Citrate thrombin': distinguishes a new product which Seegers et al [432] obtained in 1948 simply by incubating purified prothrombin in 25 percent sod citrate Several other salts (but not all) could be used and the reaction could be modified by certain diphenyl sulfones [432] It is extremely difficult to evaluate the biological significance of this very artificial method of thrombin formation However it must have biochemical significance Refractive index ultracentrifugation

small quantity (about 2%) of nitrogen not recoverable in fibrin even in optimal clotting of the highly purified fibrinogen now available

b) the *fibrinogen molecule activated by this action of thrombin* now contains some 20 30 acidic groups which can very well account for the first phases of polymerization to fibrin;

c) with prolonged action of strong thrombin e g 3 4 mg thrombin acting on 150 mg fibrin for 24 hours at 37°C complete fibrinolysis can occur (Guest and Ware [203]) associated with further small N P N increase [307] Hence

d) fibrin is just a transitory stage in the continuous degradation process of fibrinogen by thrombin

In this special way therefore we may now return to a proteolytic concept of the action of thrombin on fibrinogen

e) Other coagulants, etc. Properly purified fibrinogen solutions are quite stable and are never 'spontaneously' coagulable Whereas thrombin is the coagulant always required under physiological circumstances some other means of coagulating fibrinogen are available experimentally Examples are

1) crystalline papain [112] a proteolytic enzyme from the paw paw (papaya) fruit Unlike thrombin (usually) papain readily goes on to digest the clot
2) staphylocoagulase [186] a product of the reaction of a pro staphylocoagulase from broth cultures of certain micrococci with a plasma factor [187] which is apparently unrelated to prothrombin
3) certain snake venoms e g 3 species of Bothrops and 3 or 4 of Crotalus according to Eagle's studies [111]

True fibrin clotting should be distinguished from a variety of fibrinogen precipitations (which are reversible) and denaturations (most of which are not) The pseudo clot with ninhydrin [82] is a case in point Some of the author's [155] dark field observations of (a) thrombin (b) papain and (c) ninhydrin clots are shown in the accompanying photomicrographs (Figure 6) (III) and (IV) show the nondescript appearance of ninhydrin clots compared with the fibrillar gels formed with (I) thrombin and (II) papain Laki [275] has recently confirmed the last

4 THE CONVERSION OF PROTHROMBIN TO THROMBIN

It is with this area of the clotting field that the major investigations of this dissertation are concerned The best modern prothrombins do not change into thrombin 'spontaneously' (but cf [425]) Among the activators mentioned in the schemes of Figures 1 and 2 are:
(1) calcium (2) thromboplastic factors (3) 'accelerator' factors
(4) 'convertor' factors Noncommittal terms are used in approaching these problems with an open mind

a) Purity of prothrombin preparations. Prothrombin is difficult to purify The early preparations of Schmidt Pekelharing Hammarsten Mellanby Howell Eagle [110] and others were undoubtedly too crude when reviewed in the light of modern advances [425 276 491] Nevertheless they gave us many of the basic ideas concerning the activation

have continued to suggest that (protein) 'bound' calcium can play a role in clotting Even Quick [383 394] still retains the idea that pro thrombin is a 'calcium compound ' There is little point in arguing about this since the weight of evidence supports the conclusion that ionized calcium must participate at some point in the conversion of prothrombin to thrombin A possible reconciliation of the two view points will be suggested from data presented in this dissertation Under all ordinary conditions of activating purified or even crude pro thrombins omission of ionized calcium salt or the presence of suf ficient excess of citrate or oxalate fails to result in thrombin forma tion by thromboplastin (and adequate accelerator and convertor fac tors) The special conditions under which thrombin may be formed apparently in the absence of ionized calcium include (1) Seegers' [432] 'citrate thrombin' formation (see above) which we shall not attempt to explain; (2) experimental mixtures such as those of Milstone [339] (see p 48) and of recent workers [40] with the thromboplastin generation test (see p 48) which are best explained as 'intermediates' containing bound Ca (which however was ionized in an earlier phase)

f) <u>Thromboplastin and the activation of prothrombin</u> This topic will be explored in much detail in the body of this thesis Aqueous (saline) tissue extracts of many kinds are powerful activators of purified (and crude) prothrombins in the presence of calcium salts and adequate amounts of 'accelerator' and 'convertor' factors Puri fied cephalin seems to have thromboplastic actions but much weaker especially with Seegers' and similar prothrombins It is now gener ally regarded as an incomplete' thromboplastin Platelet suspensions also act as an 'incomplete thromboplastin So does a red cell lipid fraction [105] However when platelets or cephalin are incubated with several other plasma factors (including AHF PTC etc see later) a complete' thromboplastin is 'generated ' Modern workers are still trying to find full explanations for these experimental facts (see p 47)

g) <u>Thromboplastic enzyme,'</u> The coagulant property of weak solu tions of pancreatic trypsin which some earlier observers erroneously concluded to be a 'thrombin like action was clearly shown by Eagle & Harris [112] to be the result of some role in the activation of pro thrombin in experimental test systems Some of the author's exper iments with crystalline trypsin will be mentioned in this thesis More recently Schultze & Schwick [423] have examined extensively the con ditions under which trypsin transformation of prothrombin to thrombin can be carried out Lamy & Waugh [280] report some ultracentrifuge studies (see 'trypsin thrombin ' p 24)

The author [141] spent several years trying to establish a similar role for the natural plasma 'tryptase (fibrinolysin or plasmin) and possibly to give it an important role in the initiation of ordinary blood clotting [136] However the final outcome [476] was evidence that trypsin inhibitors could prevent the proteolytic actions of fibrinolysin without removing the thromboplastic' *effect* quite *contrary to the* findings with trypsin Pending possible reopening of this line of inves tigation it must be concluded that any thromboplastic effects of fibrin olysin preparations must be due to some unidentified impurity which they contain Ratnoff Hartmann & Conley [402] also investigated the

and electrophoretic studies on 'citrate thrombin' give evidence of an initial splitting of prothrombin into two similar (molec wt : 34 000) fractions but on dilution there appear 'aggregates' and 'disaggregates' and continuing changes over a 24 hour period [280]

(3) 'Trypsin thrombin': is also discussed in the cited reference [280] and will be mentioned again later (p 25)

These modern data are difficult to analyze but do seem to indicate that prothrombin can split up into a number of molecular fragments some of which can re aggregate probably into new agents Autoprothrombin I has been identified with proconvertin [491 425 13] while autoprothrombin II has properties resembling PTC [429] In the formation of both 'biothrombin' and 'citrate thrombin ' small but significant amounts of CCl_3COOH soluble carbohydrate and tyrosine compounds are liberated confirming the idea that thrombin forms from prothrombin as the result of some 'splitting' process [427] These very latest ideas cannot yet be integrated with the older data on mechanisms of prothrombin activation from the viewpoints which are presented in this dissertation

d) 'Prothrombinogen' (?) In 1949 Quick & Stefanini [396] claimed to distinguish between free' prothrombin and a precursor form for which they suggested the name 'prothrombinogen ' They compared their experiments with older data of Noif [351] and Bordet [55] and concluded that their prothrombinogen differed from Bordet's 'prosero zyme' in only a few particulars Only a part of the plasma prothrombin is believed by Quick to be in the precursor form and Quick & Hussey [389] (cf [154]) have recently attempted to explain the normal 'prothrombin time' of infants (see our Table LII) on the basis of a lack of prothrombinogen Few contemporary coagulationists consider that Dr Quick has enough factual evidence to support his shrewd ideas (see Macy Foundation Conferences) but Ware [491] is now inclining toward the prothrombinogen idea The present author [154] remains unconvinced

e) Calcium in relation to the conversion of prothrombin into (bio)thrombin Older plasma clotting experiments by Nordbo [352] and McLean & Hastings [325] extended the earlier idea of Sabbatini [417] that clotting depends upon calcium ions There is a definite optimum of calcium required approximately that available in the blood under physiological conditions Not merely the calcium ion concentration but the actual ion 'activity' (in terms of the Debye Huckel theory) requires consideration Nordbö particularly studied this and further pointed out that the 'available' calcium ions in a mixture of plasma oxalate (or citrate) and added calcium salt depend upon a time consuming process of attaining an equilibrium of the different forms (free Ca^{++} inactive (CaCit) anion and protein bound Ca etc) It is because of this time factor that a several fold excess of citrate or oxalate must be added to prevent clotting and conversely that much less than the stoichiometric equivalent of calcium to anticoagulant need be added to restore clotting (cited [125])

Protein bound calcium is present in the blood plasma normally and in prothrombin and other materials prepared from it unless very modern techniques of complete decalcification (e g by ion exchange resins) are used to obviate this A number of workers (cited [125])

the same factor which (a) Warner Brinkhous & Smith [497] suggested as a prothrombin 'convertibility' factor and (b) Mann et al [319] called 'co thromboplastin We prefer to adopt the names proconvertin (for the precursor) and convertin (for the activated form) as suggested by Owren [364] There are other suggested synonyms (p 20 of ref [453])

Graham & Hougie [198] report that mixture of the bloods of Alexander's original case R [9] and ours R. S. [298] results in mutual correction of the coagulation defects Together with other experimental findings this suggests that there may be more than one factor involved under the terms SPCA proconvertin factor VII stable factor etc Calcium and thromboplastin convert proconvertin to convertin and abolish the lag phase in prothrombin activation to thrombin [295]. Serum often contains much unaltered proconvertin as well as some convertin the total activity being much elevated

In deficiency of proconvertin* prothrombin conversion is very slow and inadequate Restoration by partly purified preparations from human [9] or bovine plasma (our laboratories) completely restores both rate and yield of thrombin formation Dr Lewis and the author [298] reported two cases of severe congenital 'hypoproconvertinemia' including Stuart in whom the prothrombin consumption was reduced This had not been observed in earlier case reports [9] due we believe to their being a milder degree of this defect cf [198] We have two more recent cases of VII lack (unpublished) in whom the prothrombin consumption was within normal limits Our congenital 'hypoproconvertinemics' did not respond to vitamin K_1 unlike the milder acquired hypoproconvertinemias accompanying hypoprothrombinemias in (a) vitamin K deficiency of infants (b) liver disorders or (c) after the drugs dicumarol tromexan phenylindanedione etc (author's data) confirmed by Naeye [350] The one-stage [101] assay method is satisfactory for clinical use [298] We [298] have also had some success with a two stage technique which enabled us to compute a 'proconvertin' index (see p 51) *Stuart factor was not separated however

j) Plasma factors in thromboplastin generation While tissue thromboplastin may very well play a significant role when blood is shed over injured tissues it cannot explain the good clotting which occurs in blood which has been obtained directly from a blood vessel e g by venepuncture with every precaution to avoid tissue contamination The necessary thromboplastin is generated in the blood itself by a complexity of reactions involving (1) platelets (2) several plasma factors which are present normally but deficient in hemophilia and some recently identified 'hemophilia like' conditions (3) some of the other prothrombin activators The platelet factors and the thromboplastin generation test will be discussed in a later section

(1) Hemophilia and related problems Only a few key points will be reviewed from the extensive recent literature in this field and most of the older work will be excluded

(a) Hemophilia A Notwithstanding efforts to account for the hemophiliac's clotting difficulty on the basis of some inhibitor e g the 'antithromboplastin idea of Tocantins et al [470 474 475] the major accumulation of evidence points to a specific clotting factor present

relationship between proteolytic activity of plasma and blood coagulation and in a later paper Ratnoff & Colopy [401] suggest that they may have been dealing with 'Hageman factor' (see later under Hemophilia)

h) 'Accelerator' factors in prothrombin conversion Many modern workers were fully aware that calcium and thromboplastin did not provide a sufficient answer to the question of prothrombin conversion The most significant step however came from the very extensive studies of P A Owren [361] on a human patient suffering from a peculiar bleeding disorder which he named 'parahemophilia' [362] The new factor which this patient was finally shown to lack was called 'factor V' and later 'proaccelerin' by Owren [364] Quick [384] had earlier evidence for a 'labile factor ' Another lead was the 'thromboplastin cofactor' of Fantl & Nance [119] Owren [361] also provided evidence for conversion of his 'factor V' (proaccelerin) into a 'factor VI' (accelerin) Ware & Seegers [493] prepared the new agent under the name 'accelerator globulin ' by which it is now widely known or 'AcG' for short They also found differences between 'plasma AcG' (proaccelerin or factor V) and 'serum AcG' (accelerin or factor VI) the latter being formed from the former by a trace of thrombin Stefanini & Dameshek (p 20 of ref [453]) list other possible synonyms

The present author and his colleague Dr Jessica H Lewis have studied (1) cases of congenital hypoproaccelerinemia [297]; (2) differences between proaccelerin and accelerin in the activation of prothrombin (cf [492]); (3) destruction of AcG by fibrinolysin [299]; and (4) its role in performance of the two-stage prothrombin assay and in determining prothrombin consumption in clinical cases [153] In summary:

(a) (pro)accelerin is essential for the conversion of prothrombin to thrombin by calcium and tissue thromboplastin;

(b) a trace of thrombin converts the precursor form (proaccelerin) into the active accelerin;

(c) AcG has more effect upon the thrombin yield than upon the rate of activation which is somewhat delayed when AcG is very inadequate however;

(d) satisfactory assay methods are now available both by a 'one stage' (specific) technique [395] and a 'two stage' [493] including modifications (see pp 58 59) developed in the author's laboratory for application to clinical cases of hemorrhagic disorder Plasma serum and platelets may be tested by these techniques;

(e) serum except from the ox cat or rabbit [349] is apt to be devoid of this factor because of lability and due to the fact that it is used up during clotting;

(f) AcG levels fall after hepatectomy [322] or liver injury [461]

i) 'Convertor' factors in prothrombin activation The discovery of this factor also owes much to the first clearly identified clinical case of B Alexander and colleagues [9] They named the factor SPCA (serum prothrombin conversion accelerator) and obtained evidence for a precursor (pro SPCA) [11 8] Owren was on the track of this additional factor [360 366] but it was Koller (et al [271]) who correctly reinterpreted Owren's data and named it 'Factor VII ' It is probably

and (3) others (see below) Another interesting recall is the obtaining of an 'antihemophilic globulin' from serum (rather than plasma) by the Dutch workers Bendien and Van Creveld [39] a year or two ahead of the Boston (Thorndike) group [368] Unlike AHG PTC persists in the serum since it is not completely consumed in clotting The present author suggests therefore that the Dutch workers were really dealing with hemophilia B and that their 'globulin' was a PTC preparation

(c) PTA deficiency (Hemophilia C) R L Rosenthal and co workers [413 412 414] have uncovered cases allegedly lacking a third plasmatic thromboplastic factor (PTF) which they call plasma thromboplastin antecedent (PTA) We have confirmed the defective prothrombin consumption in one of their cases

(d) 'Fourth factor' deficiency (Hemophilia D)? Spaet Aggeler & Kinsell [446] encountered another type of case with a possible fourth plasma component but did not rule out an inhibitor of PTC

(2) 'Hageman Factor.' Ratnoff & Colopy [401] observed three patients with prolonged bleeding times but without significant hemorrhagic symptoms Their studies suggest yet another factor the chief action of which is failure compared with normal plasma to accelerate the clotting of platelet poor plasma obtained for normal blood centrifuged at high speed without anticoagulant They gave it the name 'Hageman factor ' after the first patient and obtained correction of the defect with a fraction prepared from normal $BaSO_4$ adsorbed heated serum

(3) 'Factor X. Duckert Koller and colleagues [108] investigating sera from various pathological conditions in the thromboplastin generation test concluded that still another factor must be recognized ('Factor X') It is said to be deficient particularly in sera from cases with hepatitis or persons under treatment with a dicumarol derivative (Marcoumar) 'X is now used for Stuart factor (see SUPPLEMENT)

5 INHIBITOR PROBLEMS

a) Inactivators of thrombin Quoting Seegers [425] the following mechanisms participate in the removal or neutralization of thrombin (the comments are those of the present reviewer)

1) Thrombin may be adsorbed on fibrin Fibrinolysin can release it [268]
2) Large amounts of thrombin are neutralized by antithrombin This antithrombin does not require heparin for its action This is the classical antithrombin of serum or plasma [505 506] It acts over a period of time in a progressive manner [190] and is limited in degree i e a given amount of antithrombin can neutralize up to a definite limit of thrombin Morawitz [344] used the term metathrombin' for the inactive product which thrombin forms with the antithrombin of serum His claim for partial reactivation by treating serum with acid and alkali was confirmed by Gasser [184] Astrup & Darling developed a method of assaying antithrombin [21] which they later [22] distinguished from the 'thrombin inhibitor which heparin forms with some labile plasma component (co inhibitor)

in the plasma of normal persons but deficient in the hemophiliac Patek & Stetson [568] and later other workers in F H L Taylor's group at the Thorndike Memorial Laboratory of the Boston City Hospital contributed convincing evidence for this antihemophilic factor (AHF) which they called 'antihemophilic' globulin (AHG) Koller [270] lists it as 'factor VIII ' AHG accompanies fibrinogen etc in the Harvard Laboratories' plasma 'Fraction I ' It is somewhat unstable and difficult to isolate and purify Bidwell [46] in England claims a good bovine plasma fractionation but the problem needs further solution [488]

AHF is utilized or consumed in the clotting process With other factors it participates in thromboplastin generation from platelets (or cephalin) (p 46) In the natural clotting of hemophilic blood or recalcified hemophilic plasma the 'prothrombin consumption' is very defective Following this lead of Dr Brinkhous's we have also developed a method depending upon the prothrombin consumption (1 hr at 37°C) in recalcified mixtures of 'substrate' (known severe hemophilic) and normal (vs test) plasma for quantitative estimation of plasma AHF levels These tests can also be modified to assay anti AHF [152] By changing the substrate (known cases) they also assay PTC and anti PTC etc

We shall say nothing about the important familial aspects of hemophilia except to mention the very interesting work on canine hemophilia [197]

(b) PTC deficiency (Hemophilia B) In February 1952 Dr Lewis and the author [294] were investigating a 'bleeder' who seemed to be made worse instead of better by blood transfusions Our tests immediately showed that he had a circulating anticoagulant (see later) but by preparing plasma fractions free from this inhibitor an underlying clotting defect was disclosed which was definitely not hemophilia By withholding transfusions the anticoagulant gradually diminished as shown by the tests illustrated in Figure 7 clearly revealing evidence (the long recalcification clotting times in the last tests) of the basic clotting defect In April 1952 Aggeler and colleagues [5] in California published the first clearly identified case of the new 'hemophilia like' disease which they named PTC deficiency (plasma thromboplastin component) Using their techniques we quickly confirmed that our case was the identical disorder but with a new type of inhibitor complication [294] A few months later Biggs Douglas Macfarlane and colleagues [51] published an English case and from the name of of the patient coined the term 'Christmas disease ' Koller's name for PTC is 'Factor IX' [270]

An earlier lead was provided by a number of observers particularly the Argentinian Pavlovsky [370] namely that the mixing of two 'hemophilic' bloods from certain cases resulted in correction of the clotting times both in vitro and in vivo Very interestingly some of the classical 'hemophiliac' families (e g Von Tenna) extensively studied in Europe have turned out to be not what has recently been accepted as *hemophilia* but the similar *PTC deficiency (or Christmas disease)* To avoid the confusion which has resulted Dr Koller [270] of Switzerland has proposed a revision in nomenclature namely (1) Hemophilia A (the usual type); (2) Hemophilia B (PTC-deficiency);

c) Heparin co factor (heparin complement) [242] Fitzgerald & Waugh [164] have recently fractionated co factor from Cohn's plasma 'Fraction I' Their tests extend the work of Astrup & Darling [22] and do not support Klein & Seegers' [268] idea of nterference with the thrombin fibrinogen interaction (also Glazko & Ferguson's [190] 'im mediate' antithrombic action) but rather point to a definitely time consuming reaction They conclude that heparin plus cofactor inacti vates clotting by removing thrombin by direct combination Thus the reaction is 'progressive' and 'limited' Further complications arise since reaction velocity specific capacity of cofactor (units T/mg C) and extent of reversibility (using protamine) are functions of cofactor concentration These authors [164] suggest that cofactor does not occur as such in plasma but that heparin cofactors in effect repre sent *altered normal antithrombins*

Some of the author's work with heparin and cofactor etc will be presented in this thesis We shall not review the chemistry of the heparins nor the modern work on paritol treburon thrombocide sul fonated dextrans and other synthetic heparin like substances of cur rent interest particularly in the search for a cheap heparin substitute

d) Specific inhibitors of individuel clotting factors These factors being protein in nature for the most part antibodies' to individual clotting factors considered a priori are a possibility which recent investigations are beginning to support with experimental facts Such inhibitors are particularly significant when they can be demonstrated and shown to contribute to the bleeding tendency in clinical cases [453]

(1) Antiplatelet factors causing thrombocytopenia and inter fering with clotting and hemostatic functions are discussed on p 37 Normal and pathological factors which specifically inhibit one or other of the various components of the thrombin forming system are begin ning to receive serious consideration as follows:

(2) Anti AHF and anti PTC The present author and associ ates [152] presented a paper at the September 1955 Meeting of the American Physiological Society and a definitive publication will ap pear shortly By use of the prothrombin consumption test on specif ically deficient substrates (lacking AHF or PTC respectively) to which mixtures of normal and patient plasmas were added we assayed the anti AHF and anti PTC inhibitors in eight cases with hemorrhagic dis orders The routine tests on these cases showed prolonged clotting times but normal prothrombin time (Quick test) In five cases with primary hemophilia the anticoagulant could not be demonstrated by simple clot delay on mixing with normal blood or recalcified plasma but required the specific anti AHF test One hemophiliac bled to death in 18 days following a tooth extraction despite a record 400 trans fusions with whole blood plasma and antihemophilic globulin We could demonstrate removal by the potent inhibitor of the transfused AHF faster than it could be supplied In all five hemophiliacs the acquired inhibitor was specifically anti AHF whereas in the two PTC deficients it was anti PTC The eighth case of 'idiopathic' in a female and showed a very high titer of anti AHF as well as an apparently high titer of anti PTC The last might be questioned on technical consider ations

Inhibitors of the above type have been reported in a very small mi nority of cases of hemophilia PTC deficiency; certain allergies; some

They state [22] while normal antithrombin seems to combine with thrombin to form an undissociable compound the compound between thrombin inhibitor and thrombin as well as the compound between heparin and thrombin coinhibitor seems to be highly dissociable ' That fat solvents can inactivate antithrombin was noted in the early literature chloroform especially being used for this purpose [351] although it has the disadvantage of also destroying thrombin [476] Seegers et al [433a] have done many experiments with ether especially to remove this antithrombin from defibrinated plasma Sternberger [456] used alcohol and has obtained *some very provocative results* [457] *Fibrinolysin will not work* [476]

3) Heparin co factor together with heparin interferes with the reaction of thrombin and fibrinogen This will be discussed further in a subsequent paragraph (p 31)

'4) Antithrombin accelerator activity arises during the clotting process and also neutralizes thrombin ' Seegers' [425] new agent (AA = antithrombin accelerator) refers to the antithrombin demonstrable after ether extraction of defibrinated plasma To it is attributed the disappearance of thrombin formed therein after activation of the prothrombin by calcium (lung) thromboplastin and (platelet) AcG the latter reagents not being the source of the alleged AA factor Further (Seegers [425]): There is an inhibitor of the antithrombin accelerator action which we call antithrombin accelerator inhibitor AA inhibitor is removed by $BaCO_3$ adsorption and is recoverable in the citrate eluate These new ideas of Seegers are highly provocative particularly in regard to his suggestion that AA inhibitor is proconvertin or associated with proconvertin in partially purified materials

b) Heparin [242 382 151a 151 22] It is now generally agreed that little if any heparin is present in the circulating blood normally [249 61] The protamine titration method [250] of measuring 'heparin like' activity in plasma is subject to questions of interpretation but does agree with anticoagulant tests e g prolongation of clotting-times of whole blood [15] or recalcified plasma routine in author's laboratory p 61 when heparin is known to be present This is true both in vitro and in vivo In vivo however heparin may be removed from the circulation by (a) renal excretion [254] (b) metabolic alteration or (c) a heparinase enzyme [248 252] Chemical extraction and assay of the metachromatic color reaction of heparin with azure dyes are the most convincing methods [342] They have recently been used in conjunction with paper chromatography [36] Wilander's work [501] concerning the origin of heparin from the tissue basophils or Ehrlich mast cells is now generally accepted These mast cells appear to break down and release significant amounts of heparin (proved conclusively by chemical extraction) in certain pathological conditions particularly those involving an anaphylactoid type of reaction [255] They also yield histamine [195] which probably forms a complex with heparin [403] Rocha e Silva [407] implicates a fibrinolytic enzyme activation and finds histamine release to be an accompaniment Hyper heparinemia can cause bleeding disorders [448]

1) it is a constituent of normal (platelet free) plasma
2) it inhibits added cephalin or tissue thromboplastin but requires a special series of minimal plasma dilutions in order to demonstrate and assay its effects
3) it is less effective in the presence of wettable surfaces or after the blood has clotted
4) it is somewhat species specific and does not affect the thromboplastic action of Russell's viper venom
5) it can be concentrated in certain plasma fractions and in lipid rich tissue extracts Both these points were con firmed by Overman [358 359]
6) it is increased in cases of hemophilia [475]
7) it is also increased in post irradiation hemorrhage [471]

Dr Tocantins is a very able and careful worker but there is now considerable doubt as to the significance of the data under discussion One group [196] raises many objections including questions as to the control of ionic strengths in the limited dilution experiments

e) Summary of the inhibitor problems and their clinical signifi cance From the foregoing brief review it is evident that the inhibit ors are a little known and difficult area in the coagulation field More knowledge is needed because of their importance in occasional clinical cases Valid information has begun to accumulate as the result of the devising of specific testing techniques In some cases the diagnostic question is settled The therapeutic goal is elusive however Adrenal cortical hormones failed in an extensive trial on two of our clinical cases The relationship of inhibitor appearance to transfusions neces sitates a searching review of this form of therapy There is need for more rapid advance in the difficult field of fractionation of the individ ual clotting factors from the blood in practical preparations which can be used for rational replacement therapy

BLOOD PLATELETS

A HISTORICAL

Several careful microscopists in the early nineteenth century must be credited with the discovery of the blood platelets although they were ignorant of their true nature and functions Most of these workers are cited in a 1934 review of the history of haematology by Sir Humphrey Rolleston [408]

Alexander Donne (1842) [106] termed them globulins' (a term still used in the French literature) but probably confused them with fatty particles of the chyle Addison (1841) possibly Wagner (1842) and certainly Andral (1843) [17] observed them in commencing clotting of the blood Andral thought them to be 'fibrin molecules ' as did Fr Simon G Zimmerman (1848 60) [516 517] also cited Gerber's 'free nuclei' and Fr Arnold s *Elementarkorperchen* as well as Simon s term 'Kugelchen Zimmerman used Simon s technic of re ceiving blood into potassium ferrocyanide and delayed clotting enough to permit investigation of the supernatant still more or less turbid fluid He also could receive the blood into neutral or carbonic acid salts In slowly clotting bloods he clearly noted the 'little bodies'

systemic collagen diseases [175 453]; and questionably associated with pregnancy History of previous transfusion therapy can usually be elicited In one of our PTC cases the evidence was very clear that transfusions led to the recurrence of high titers of the inhibitor In this case and in the 'idiopathic' female electrophoretic analysis re vealed an abnormal increase in serum gamma globulin This is further evidence that the inhibitor is acquired by some unusual type of 'immunity reaction ' However the result in causing transfusions to become harmful instead of helpful complicates the clinical control of the bleeding problem

(3) Anti(pro)convertin (Factor VII inhibitor) Wagner et al [487] have obtained certain fractions from normal dog plasma which specifically inhibit active convertin Jurgens [259] has prepared a similar agent from normal human serum and suggests that it has cer tain properties in common with plasma and serum antithrombin

(4) Anti(pro)accelerin (Factor V inhibitor) has been questionably reported by Hörder [223] in a case with a bleeding disorder It is said to be 'lipoid like' and to cause the factor V deficiency which explains the bleeding syndrome in the case cited *

(5) Antithromboplastin (A) in the sense of a circulating in hibitor *of added tissue thromboplastin in cases with a bleeding ten dency* is suggested in several accounts referred to on p 209 of the new text by Stefanini & Dameshek [453] The present author suggests that the positive identification of a genuine antithromboplastin must fulfill certain criteria:

(a) it must specifically inhibit added tissue i e 'complete' thromboplastin e g in the one-stage prothrombin time (Quick) test

(b) *there should be no alternative explanation possible on* the basis of inhibition or lack of some other factor in the thrombin forming system

Hougie [226] has already pointed out some of the interpretive difficul ties For instance if the inhibition is demonstrable only at high dilu tions of the added thromboplastin it might be due merely to lack or inhibition of the plasmatic thromboplastic components (AHF PTC etc) In fact the test might be no more in essence than the perform ing of the partial thromboplastin test of Langdell et al [282] These authors indeed use it to assay the lack of AHF When Fanti & Nance [120] investigated an Australian woman and reported inhibition of hu man brain thromboplastin but not that of other species they might simply have been observing the influence of dilution on two different reagents which did not have true equivalency of thromboplastic potency in the first place

Among the several cases cited [453] the patient tested by Char gaff & West [81] and later restudied by Conley *et al* [92] *shows* that many turn out to be anti AHF anti PTC etc AcG controls are im portant also

Antithromboplastin (B) in the sense of Tocantins et al [474] re fers to an alleged plasma factor for which the following claims are made:

*Ferguson et al described an unequivocal case of anti AcG (Proc Soc. Exp. Biol. & Med. 95: 567 1957)

1 PLATELETS IN THE BLOOD UNDER NORMAL AND THROMBOTIC CONDITIONS

Many workers have confirmed and extended the observations of Bizzozero [54] and Eberth & Schimmelbusch [113] Several labora tories have shown fine cinematograph films of the circulation in small vessels including platelet observations e g those of (1) M Knisely [269] (formerly of Chicago now at Charleston S C); (2) the research team at the Hoffman La Roche Swiss laboratories; (3) the Boston Uni versity group under G P Fulton and B R Lutz [41]; (4) Jean Hughes at Liege

In vivo platelets are tiny little discs which appear lenticular or 'batonnet like'* when viewed edgewise Endothelial injury causes leukocytes and platelets to 'marginate' from the axial stream to the 'skimmed' plasma layer closest to the blood vessel wall Platelet plugs can be seen to form and seal off microscopic holes in an injured vessel particularly in venules The platelets adhere individually and to other platelets They also break away one by one or in clumps of varying size These may be arrested temporarily or more perman ently further along the course of the circulation

In observations on the formation of cellular thrombi in the frog mesentery on pricking the capillary endothelium with a microneedle (Kite Chambers micromanipulator) the author [127] noted the sig nificance of rate of blood flow In slower rates of flow all the corpus cular elements enter into the thrombus formation and the red cells being the most numerous largely preponderate In more rapid rates of flow it is the stickiest elements which have the best change of re maining adherent Hence the distinction between 'white (thrombocyte or platelet) thrombi and red' or 'mixed' thrombi long recognized by pathologists Hayem s (1889) term clou hemostatique and the German 'Blutstillenden Keil' are highly descriptive (p 82 of Quick [383]) Wharton Jones (1851) Mantegazza (1869) and especially Zahn (1872) [513] preceded Bizzozero [54] in demonstrating the importance of white thrombi for controlling hemorrhage after vessel injury

2 VISCOUS METAMORPHOSIS AND PLATELET ADHESIVENESS

The thrombotic platelet mass later evinces a hyaline or granular appearance for which the term 'viscous metamorphosis' was coined by Ebert & Schimmelbusch [113] and later used by J H Wright and G R Minor [511] At one of the Macy Conferences [509] Helen P Wright reviewed this topic and described her quantitative approach based upon rotating the platelet containing fluids in glass tubes for

*Few workers have bothered to check the term batonnet as originally used by Aynaud [24] N Rosenthal in H Downey's Hand book of Hematology [411] says baton like and the latest note of the Boston workers [41] uses bayonet like Actually Aynaud compared this platelet appearance to the little stick with tapered ends which is used in the childish game that the English call tip cat the Americans mumbly peg and the South Africans kennetjie

(kleine Korperchen) before fibrin appeared and described the latter as forming homogeneous masses or threads often radiating from the 'little bodies' or in some cases from leukocytes He concluded in favor of the idea that " these little bodies already exist in the circulating blood ' As they resisted ether and warming Zimmerman showed they were not fatty globules but an organic (vesicular) formation ('organische Bildungen mit Inhalt gefullte kleine Bläschen') Zimmerman also studied birds and amphibia and decribed the nucleated cells (distinguishable from leukocytes but confused with erythrocytes) which were termed 'thrombocytes' by later writers [100] He discussed their formation in terms of Schwann's "cell theory' and suggested a lymphatic origin

In 1865 Max Schultze again noted them in human bloods as did Vulpian (1872) and Riess (1872) who thought they were remains of disintegrated leukocytes In 1874 William Osler [357] gave another classical description which distinguished them from bacterial micro organisms In 1877 Hayem [213 214] called them 'hematoblasts' or the 'third' (formed) element of the blood and insisted that they were precursors of the erythrocytes a view which he maintained as late as 1923 [216] While much of the confusion of platelets with the other formed elements may be blamed upon poorly controlled observation in vitro two more recently identified phenomena may be suggested as sources of error (1) living leukocytes can lose fragments during ob servation (dark field) on glass slides in a shed blood drop [122]; (2) erythroplastid [116] formation can occasionally be observed under similar conditions from certain erythrocytes or their precursors

Figure 8 shows one of a series of 14 photomicrographs taken by a former colleague (Dr P H Ralph) over a 2 hr period in an observation of an erythroplastid forming from a bone marrow erythroblast in a case of Hodgkin's disease

Bizzozero's [54] work was most significant He described platelets in the circulation in the mesenteric vessels of rabbits and guinea pigs and later in the intact bat's wing He demonstrated their adhesive quality their participation in thrombi and their role in the coagulation of the blood

Lowit [311] tried hard to prove platelets to be mere artifacts e g leukocyte fragments but Bizzozero's data settled the controversy Eberth & Schimmelbusch (1886) gave a classical description [113] of experimental thrombosis including the role of the platelets and of fibrin in the transilluminated mesentery

J H Wright [510] identified the origin of the mammalian blood platelet from cytoplasmic fragments of the megakaryocytes of the bone marrow He also showed the phylogenetic relationship to the thrombocyte of the lower animals which serves similar physiological functions in these orders Dr W H Howell showed me his beautiful tissue preparations obtained for a 1937 paper [241] in which he stated: platelets represent a solid secretion from a unicellular gland the megakaryocyte This is a good way to emphasize the conception of a dual nature of platelets namely partly like a cellular element and partly as 'amorphous' material contributing to the chemistry of clotting etc

and colleagues [45] These Toronto workers inserted a glass cham ber between an artery and vein in an experimental animal and observed platelet agglutination and adherence to the flat surfaced glass chamber microscopically The cinematograph film of these observations was re exhibited at the Macy conference (1949) Heparin caused a great reduc tion or even absence of observable platelet agglutination Dr Best and others [161] believe that some heparin preparations contain impuri ties which could explain the findings of Copley et al

4 PLATELET ANTISERA PLATELET GROUPS AND THE THROMBOCYTOPENIA PROBLEM

Following the 1905 suggestion of Marino Bedson [33 34 35] in- jected rabbit platelets into guinea pigs and obtained a platelet anti serum which caused thrombocytopenia and endothelial (capillary) dam age when injected into 'non sensitized' animals Ackroyd [1] has re cently reviewed the problem of "platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura He includes:

a) the 'abnormal splenic secretion' theory of Frank (1925);
b) the efforts of Troland & Lee (1938) and others to obtain spleen extracts ('thrombocytopen') allegedly capable of reproducing the human thrombocytopenic condition in experimental animals;
c) the demonstration by Evans Duane et al (1949 51) of a 'throm bocyte agglutinating factor' in the sera of some ITP (<u>I</u>diopathic <u>t</u>hrombocyto<u>p</u>enic) patients;
d) several reports that transfused platelets survive for shorter periods in ITP cases;
e) Harrington's [207] convincing experiments in which by inject ing himself with plasma from an ITP case he lowered his platelet count to dangerously low levels and was fortunate to recover;
f) confirmatory repetition of this extremely carefully conducted by Stefanini et al [454];
g) Tullis' [478] finding that 9 out of 18 sera from ITP cases caused agglutination and lysis of normal platelets in the presence of complement;
h) demonstration by both Harrington s [208] and Stefanini s [455] groups that platelet agglutinins could occur in some patients after pregnancy or transfusions; or
i) even in some individuals who have never been pregnant or trans fused [455]; and
j) the confusion due to demonstrations [451 502] of fall in platelet count following transfusions of <u>normal</u> blood etc

This whole problem is beset with technical and interpretative difficul ties but the following conclusions represent current thinking

1) antiplatelet agglutinins and lysins occur in humans sometimes without but usually with thrombocytopenic purpura;
2) there are platelet 'groups' or 'types ' analogous to those en countered with red cells;
3) 'acquired' cases of thrombocytopenia often have a demonstrable cause of agglutinin production Ackroyd [1] has made some very clear cut studies of drug induced (sedormid; quinidine)

definite time periods and comparing the platelet count (hemocytometry) with a vaselined-tube control She reduced 'platelet adhesiveness' by heparin and chlorazole dyes in vitro and by dicumarol in vivo Conversely adhesiveness was increased after operations or parturition at periods when the platelet count was also elevated and many young forms were circulating Similar findings were reported for the thrombocytosis following injections of adrenalin or of pyridine Splenectomy gave similar results but now there was little further effect from pyridine Moolten & Vroman [343] with an improved technique failed to confirm Helen Wright's suggestion of an increased platelet adhesiveness in hemophilia Her idea in this disease is also contrary to observations of many other workers Dr Wright's interpretations included the following (with the present reviewer's parentheses): the factors which appear to maintain the various (formed) elements in suspension are first their surface charges (p 174 of Tocantins [469]) which are of the same (electrical) sign; and second their constant motion both as a mass and relative to one another ' 'It is believed moreover that the surfaces of the formed blood elements carry variable amounts of adsorbed water and that this helps to render them stable in suspension as well as augmenting the viscosity of the blood '

their surface reactions may be in part dependent on some of the metabolic activities of the cell The platelet surface may be modified by substances normally (fibrinogen globulin) or abnormally (immune globulins of anti platelet sera) present in the blood Adhesiveness must also be related to alteration in the wettability or stickiness of injured vascular endothelium [419] It may be recalled that Roskam [415] collected many data leading to the suggestion that platelets are surrounded by a firmly adsorbed protein film to which many of their properties may be due That this is not necessarily fibrin or fibrinogen however is indicated by the normal platelet adhesiveness etc in a case of congenital afibrinogenemia studied by Pinniger & Prunty [376] and confirmatory observations by Alexander et al [10] and others [296] cited [7]

3 PLATELET 'AGGLUTINATION (NORMAL)

According to Aynaud [24 25 26 27] platelets suspended in plasma may be agglutinated by a wide variety of substances e g gelatine egg albumin peptone gums lecithin heavy metals dyes and surface tension lowering agents such as ricin sod taurocholate and saponin The peptone plasma experiments were particularly interesting as showing that platelet agglutination could be independent of clotting Tocantins [469] reviewed many of the older papers dealing with platelet agglutination under various experimental conditions and in numerous clinical disorders A number of modern workers have restudied some of these problems on well washed (? plasma free) platelets especially aided by the silicone technique (see pp 67-68)

Copley & Robb [93] observed that washed platelets could be agglutinated by adding plasma or serum especially the latter and also by 'purified' preparations of globulin prothrombin thrombin placental tissue juice and plasma with a high concentration of heparin (!) This last finding was unexpected and in conflict with the data of C H Best

and colleagues [45] These Toronto workers inserted a glass chamber between an artery and vein in an experimental animal and observed platelet agglutination and adherence to the flat surfaced glass chamber microscopically The cinematograph film of these observations was re exhibited at the Macy conference (1949) Heparin caused a great reduc tion or even absence of observable platelet agglutination Dr Best and others [161] believe that some heparin preparations contain impuri ties which could explain the findings of Copley et al

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1 RELATION OF PLATELETS TO HEMOSTASIS ETC

As noted previously platelets play a key role *in thrombosis* and embolism besides acting as a defensive mechanism against blood loss Pure platelet or 'white' thrombi are relatively rare and the usual thrombus is 'red' or 'mixed ' with the bulk and numbers of erythrocytes preponderating Moreover clotting is initiated about the time (Erkelens' thesis [118]) that platelets adhere and begin to disintegrate Hence a thrombus is also rich in fibrin which forms a supportive meshwork and buttressing that adds greatly to the hemostatic effectiveness In the clinical 'bleeding time test' (e g methods of Duke [109] or Ivy [244]) defects of platelet or vascular function rather than of clotting function are causative of prolongation of the bleeding The bleeding time is not infrequently normal or nearly so in cases of hemophilia for instance Conversely the whole blood 'clotting time' test (e g Lee-White method [286]) is often normal in thrombocytopenics and primary vascular diseases

Since hemorrhagic disorders [453] can accompany (1) vascular abnormalities (2) platelet deficiencies quantitative (thrombocytopenic) or qualitative (thrombocytopathic or thrombasthenic) or (3) coagulation inadequacies it is evident that all three normally share in the defensive hemostatic mechanisms

Vascular anomalies are beyond the scope of the present thesis Just how important the contributions of the first two factors may be is emphasized by known cases of afibrinogenemia [7] who lack the essential clotting mechanism These cases do have bleeding problems but often manage much better than say a severe hemophiliac May it not be that certain factor(s) of the thrombin forming mechanisms are also necessary for some platelet functions?

Serotonin, Marjorie B Zucker [518] performed experiments on the formation of the hemostatic plug which appears after cutting a (small) blood vessel in the rat Noting a frequent vasoconstrictor effect at the time of platelet deposition Dr Zucker investigated this additional phenomenon and used an ear vessel perfusion technique for bioassay of the 'platelet vasoconstrictor' factor The consensus of a number of workers [367] on this problem is that disintegrating platelets liberate *5-hydroxytryptamine* [399] *which reacts with some* plasma factor to become 'serum serotonin (probably 5 hydroxy tryptamine creatinine sulfate) previously recognized by Rapport et al [400] We have some unpublished data showing low serum serotonin (platelet vasoconstrictor) values in cases with hemophilia and other severe clotting disorders The suggestion (p 38) is that thrombin (or some factor in the thrombin forming system) is needed for platelet 'release of 5 hydroxytryptamine

2 THROMBOCYTES

The thrombocytes serve the hemostatic functions in lower animals e g birds fish reptiles amphibia etc much as the platelets do in mammals [185 189] That they are not quite as efficient in some ways

thrombocytopenias showing that both drug and specific antibody are needed for the platelet-destroying action;

4) complement is involved in the platelet lysis as it is in hemolytic and bacteriolytic immunity reactions

5 IN VITRO 'ALTERATION' OF BLOOD PLATELETS

In 1873 Ranvier [397] observed the microscopic changes of platelets and formation of fibrin in a drop of shed blood He described alterations in the shape of the platelets and in "the refraction of their granulations ' However his most significant observations were: (1) fibrin needles are often seen first at the edges of platelets or platelet debris; (2) platelet 'granulations' occur at the intersecting nodes of the fibrin network

Aynaud (see p 36) made a classical series of studies of platelet alterations in vitro which have largely been neglected by more recent workers in this field However they formed the chief background for the present author's observations [124] which are embodied in this thesis With extraordinarily skillful use simply of paraffined equipment the centrifuge and the microscope Aynaud described the sequence of alterations from the original disc or batônnet (v p 35) form to the spiculated 'stellate ' swollen and granular (disintegration) forms The controlling factors studied by this worker include (1) surface (2) temperature (3)dilution of plasma (with citrate saline) (4) the decalcifying (citrate etc) anticoagulants and (5) many experimental additives (see previously)

Fonio & Schwendener [171] used $MgSO_4$ plasma to observe various platelet alterations some of them bizarre

Zucker [518] confirming unpublished observations in the present author's laboratory agrees that even siliconed surfaces which are our best non wettable surface to date fail to prevent but merely retard morphological changes in the platelets held in citrated plasma The decalcifying ion exchange resins e g Amberlite or Dowex '50' do not help In fact most recent workers find these unsuitable for platelet recovery and prefer the strong Ca binding 'chelating' agent EDTA (sodium ethylene diamine tetra acetate also called Sequestrene or Versene) ref Tullis [479]

$CaCl_2$ was not found to have much influence on the swelling etc shown by washed (x3) platelets in buffered saline (Milstone [340]) Whether or not thrombin can produce platelet alterations as claimed by Zatti [515] and Fonio [169] is still an unsettled question (Zucker [518]) but is probably true and this may account for some new facts such as low serotonin assays in severe *clotting disorders (Dr J H* Lewis the author and M B Zucker unpublished data)

B PLATELET FUNCTIONS

For the purposes of this thesis it is not necessary to review some platelet functions (e g metabolism; 'platelet loading'; etc [469]) Included are those concerned with hemostasis and allied functions

' Character of the clot its firmness rigidity and elasticity depend nearly exclusively on the fibrin concentration and the occur rence or absence of fibrinolysis They are completely independent of platelets Fibrinolysis and clot retraction are independent proces ses ' Clot retraction is due to a special function of platelets viscous metamorphosis leads to the formation of long strands or pseudopodia of cytoplasm connecting individual platelets or platelet masses These strands subsequently contract producing a patchy fusion of platelets throughout the clot As the platelets are adherent to the fibrin network of the clot the contraction of the cytoplasmic strands and the merging of the platelets result in a contraction of the whole fibrin clot with a squeezing out of the serum and a few of the red and white cells enmeshed in the fibrin network

From the delicate platelet mechanism producing clot retrac tion it would be expected that the force exerted by the process is very small By a simple experiment (p 46 [70]) this assumption is shown to be correct; the force (about 19 mm H_2O) is so insignificant that it becomes extremely unlikely that clot retraction serves any physiolog ical purpose in mammalian life It can certainly not draw together the walls of even the smallest vessels

In the crab clot formation and retraction take place in the complete absence of fibrin and are entirely cellular functions It is suggested that the process is a redundant phylogenetic relic of no importance in higher animal life

A critical review of Budtz Olsen's work will not be attempted here but the present writer would raise some questions e g :

(1) Platelets don t retract clots formed from fibrinogen with throm bin but only with 'thrombin forming coagulant mixtures The present author s experience is otherwise
(2) Clot retraction cannot draw together small vessel walls Tocan tins [468] who is cited by Budtz Olsen collapsed cellophane tubing by clot retraction
(3) Fibrinogen concentration is the chief determinant of size of fi brin mesh and hence of capillary forces There are many dis crepancies between the ineffectiveness of certain agents in modifying Budtz Olsen's clot retraction measurements and data of physico chemical and electromicroscopy studies which indi cate they can greatly alter the fibrillar composition of the fibrin clot
(4) Can fibrinolysis be critically ruled out in connection with clot retraction? (See below)
(5) Accepting Budtz Olsen's worthy critical review of Glanzmann's (1918) [188] case data on hereditary hemorrhagic thrombas thenia there are contrary to the South African s opinion un doubted cases of platelet anomalies (thrombocytopathias) in which these elements are deficient in some retractor factor (? Glanzmann's retraktozyme') Such platelets can be isolated and compared with normal platelets shown unable to induce clot retraction in normal platelet free plasma [453] Such a case will be described in the present thesis

b) <u>Role of platelets</u>, Budtz Olsen [70] refers to Thackrah (1819) and others whose work clearly indicates that the fact of deficient clot

is suggested by the relative ease of preparing incoagulable or very slowly clotting plasmas from the thrombocyte possessing bloods by careful collection and centrifuging perhaps even without use of anti coagulants This is very difficult in mammals even with use of anti coagulants because of the ease of platelet breakdown There are species differences however and equine blood has been used by a number of investigators [24]

3 PLATELETS AND CLOT RETRACTION

a) Clot retraction In 1951 O E Budtz Olsen [70] of Cape Town published an outstanding monograph on this subject with a very exten sive bibliography His theoretical approach may be quoted verbatim:

The blood clot consists of a three dimensional network of fibrin threads ' The serum is held in this network by capillary forces When the serum artificially is pressed out of the meshes the network collapses and does not again expand when immerged in serum due to adhesion between the individual fibers This can be seen by manipulation of the network under the microscope The capillary force holding the serum in the clot might conveniently be expressed by this formula:

$$\text{Force:}\ \frac{2y\cos\theta}{d\,g\,r}$$

where y: the surface tension of the serum
θ the angle of contact of the serum with the fibrin which is an expression of the wettability of fibrin
d: the density of the serum
g: the force of gravity
r: the width of the mesh

which is simply the formula for any fluid in any capillary tube Consideration of the force of gravity by this author is a new contribution

When the clot is placed in rigid structures like glass tubes gravity tends to draw the serum out of the fibrin network against the capillary forces holding it back; an artificial 'clot retraction' may thus be pro duced To avoid this Budtz Olsen designed an ingenious and simple method (' the suspended clot method') Under constant (37°C) temper ature conditions 5 ml of blood (or test mixture) are floated free from the influence of gravity in an inert oil mixture The serum expressed during retraction of the clot floats to the surface of the denser oil mix ture and can be measured It is shown that the size of the meshes in the fibrin network and therefore the capillary forces holding the serum in the clot depends mainly on the concentration of fibrinogen in the plasma " 'It unavoidably follows that clot retraction is inversely proportional to the fibrinogen concentration of major influence on clot retraction is the volume of red and white cells in the blood These cells play a passive obstructive role and it is demonstrated experi mentally that an almost linear inverse relationship exists between the volume of packed cells and the clot retraction correction can be achieved by simply adding the haematocrit to the observed clot retrac tion ' 'Rigid control of the temperature is essential in all work on clot retraction

in the most recent work [316] to present conclusive evidence to rule out all possibility of traces of active fibrinolysin being present or formed in the test systems The present author does not insist on 'the fibrinolytic theory ' but merely believes that it should be convincingly ruled out

This being so it is appropriate to consider some peculiar 'retractility' of platelet 'processes ' This will be done in the present thesis especially in terms of lipid water phase relations It can be stated with fair certainty however that platelets do not extend and withdraw true 'pseudopods comparable to those in leukocytes etc Budtz Olsen's data are very weak on this point This author however may be referred to for a good comparison between physical 'syneresis' of certain gels and the 'clot retraction' phenomenon The differences are numerous and support the conclusion: ' the fundamental mechanism of the two processes is probably entirely different

4 CLOTTING FACTORS IN BLOOD PLATELETS

a) Need of platelets for blood clotting There was much doubt about the blood platelets being essential for normal blood clotting until Brinkhous [64] using the newly introduced siliconed surface glassware of Jaques et al [251 288] showed that plasma recalcification clotting times and prothrombin consumption (utilization during clotting) were delayed the more the platelet count was reduced by centrifugation Successful experiments in obtaining a non clotting platelet free plasma will be cited (pp 67 68) in the body of this thesis The thromboplastin generation test is discussed on p 46

b) Erroneous ideas In the earlier literature about what the platelets can contribute to the blood clotting mechanism appear the following:

1) platelets act as a complete 'coagulant' for fibrinogen (a) in the presence of calcium by allegedly providing a tissue fibrinogen (Wooldridge [508] 1886; Mills [338] 1930) (b) in still earlier ideas of a similar kind platelets were not separated from the 'white corpuscles but only from red cells (Mandl 1842; Buchanan 1843 [68] cited by Anderson [16] 1844)
2) platelets contain prothrombin (Morawitz [346] 1905 Bayne Jones [32] 1912 Christie Davies & Stewart [85] 1927; Fuchs [177] 1930; Howell [238] 1935 cited by Tocantins [469] 1938) Disproved by Bordet [55] Mills [338] and Eagle [110]
3) platelets directly activate prothrombin through their zymoplastic substance (A Schmidt [420 421])
4) abnormal 'stability' of platelets is the cause of hemophilia (Sahli [418] 1905; Minot & Lee [341] 1916; Morawitz [347] 1925; Howell & Cekada [240] 1926 Christie Davies & Stewart [85] 1927; Fonio [169] 1932) See present thesis Hayem [216] 1923 suggested that whatever is responsible for the stability of hemophilic plasma also accounts for the stability of platelets in that plasma

c) Platelet thromboplastic' factor (prothrombin 'activator') Bordet & Delange [57] 1912 implicated platelets as one source of their

retraction in association with certain purpuras was known at the beginning of the nineteenth century Hayem [215] in 1896 was the first to correlate poor clot retraction in purpura with definite reduction in the number of blood platelets Among workers who have experimentally reduced platelet counts by centrifuging plasma and carefully correlated with clot retraction data we may mention Tocantins [467]

LeSourd & Pagniez [289] Bordet & Delange [57] Opitz & Matz dorff [356] and others [70] have recorded inability to obtain clot re traction with platelet 'extractives ' but only with 'intact' platelets In recent electron microscopy studies Bessis & Burstein [44 43] ob served adherence of fibrin filaments only to speculated 'altering' platelets and not to 'disintegrated' forms Glanzmann's [188] sugges tion of a 'retraktozyme' (enzyme like factor in platelets) has there fore not gained acceptance until recently when Fonio [170] claimed separation of the outer 'hyalomere' from the central 'granulomere' of platelets by differential centrifugation and the identification of 'retrak tozyme' with the hyalomere fraction Very recently 1956 Magalini & Stefanini [316] claim isolation of a 'retractin' both from platelets and from blood-free tissues Their best method is to disintegrate and ex tract with ether water (65:35) and then deposit in the cold at 20°C *Thomboplastic factor is eliminated by heating for two hours at 56°C* and subsequent storage at 20°C Tests for vasoconstrictor factor (5-hydroxytryptamine) are also negative They conclude: ' it could represent a lipoid substance '

How do platelets bring about clot-retraction? The weakness of the force involved in clot-retraction is stressed by Budtz Olsen as by previous workers While it is usually sufficient to detach the fibrin strands from adherence to glass this may not be the case in some in stances Fibrin is more adherent to collodion [220] and Tocantins [467] observed failure of retraction in such vessels However clot retraction is not significantly better in non wettable siliconed tubes Budtz Olsen reviews much of the older literature (e g Fuchs [179]) on the influence of the containing vessel's surface on clot retraction Although he cites their paper in another connection (temperature ef fect) he doesn't include a very interesting experiment of Lampert & Ott [279] which the present writer has frequently reproduced for the the benefit of his students Thus if blood is allowed to clot in a wide test tube paraffined down <u>half</u> of its side the retraction of the clot will subsequently be away from the glass and <u>to</u> the paraffin That fibrin is less adherent to paraffin than glass was shown by Fuchs (1931) and many workers have used paraffined vessels in clotting ex periments because of its 'non wettability ' It is difficult to explain the Lampert & Ott experiment except on the basis that the glass in some way activates' the retraction producing factor

Observing certain similarities between retraction e g of plasma clots containing platelets and thrombin fibrinogen clots which do not normally retract but can be made to withdraw from the glass tube and 'clump' when known to contain <u>very small</u> amounts of trypsin or fibrin olysin Ferguson & Erickson [149] revived the idea [219] that clot re traction may be related to fibrinolysis This suggestion is not easily dismissed by the arguments of Budtz Olsen whose experiments give no heed to the <u>quantity</u> of fibrinolytic enzyme It may also be necessary

thrombin formation without affecting the quantity produced However Eagle may not have tested sufficiently small amounts His weakest cephalin (0 0001 cc of 0 1" suspension) does not quite reach '100"' activation in his Figure 3 [110]

(2) Prothrombin prepared from hemophilic plasma and compared with a similar preparation from normal plasma showed in the presence of a concentrated platelet suspension considerably slower thrombin formation but a normal (100") eventual yield With calcium alone Eagle claimed ultimately normal' thrombin yields (actually his curves Fig 1 ref [110] show 80 90") but very slow rates particularly in the hemophilic Simple plasma recalcification clotting times showed that hemophilic platelets were as good as normal platelets in accelerating clotting in a series of strengths of the platelet suspensions in both (a) normal plasma and (b) hemophilic plasma However the stronger platelet suspension (both types) worked less well on hemophilic plasma and the weakest platelet strength did not work at all In comparable experiments cephalin speeded up the clotting of normal recalcified plasmas but had so little effect on the hemophilic (Table III of [110]) as had previously been shown by Mills [333] that Eagle dismissed it as unaccountably ineffective

Addis [3] had demonstrated in 1911 that hemophilic plasma was not deficient in prothrombin but that its conversion to thrombin was delayed which led him to the hypothetical suggestion of some qualitative defect in the hemophilic prothrombin Addis prepared 'prothrombin' by a method similar to that later used by Eagle and Howell [238] pointed out that both studies could be subjected to the same criticism namely that their prothrombin contained thromboplastin and that this might be what is lacking in hemophilia Using his acetone method Howell with Cekada [240] could not find any difference in quantity or reactivity between the prothrombins of normal and hemophilic blood Howell prepared a plasma thromboplastin and found less of it in hemophilic plasma Purified preparations (after glycerol extraction) were said to be protein free (ninhydrin and biuret reaction) but Howell could not obtain enough material for conclusive analyses and turned to the study of lung thromboplastin also known to be able experimentally to restore normal clotting times in hemophilic plasma independent of platelets Howell did think that his plasma thromboplastin' might come from platelets and he was able to prepare active thromboplastic material from platelets both normal and hemophilic (p 19 of ref [240])

Fonio [169] by cold centrifugation in paraffined tubes was able to obtain hemophilic plasma which clotted very slowly (4 8 hours at room temperature) but rapidly on adding washed platelet suspension from normal blood or less rapidly with washed hemophilic platelets

Other early workers including those we have dismissed under erroneous ideas argued for more stable or less active platelets in hemophilia but it is not profitable to go into this any further All recent work supports the conclusion that it is not the platelet which is at fault in hemophilia but some plasma factor (AHF) which works with platelets to generate thromboplastin' for participation in the activation of prothrombin to thrombin (see p 46)

e) Platelets and prothrombin consumption In 1939 Brinkhous [63]

tissue clot accelerating principle or 'cytozyme' and extracted a thermo stable lipid material soluble in alcohol ether and chloroform but little soluble in acetone which agreed with Howell's [231] 1912 iden tification of the thromboplastic factor as cephalin Because cephalin was less potent than crude platelet (or tissue) extracts Howell [231] and Rumpf [416] believed the true thromboplastin to be a cephalin protein (i e lipoprotein) complex ' Eagle [110] also noted that the lipid extract from platelets had only about 1/10 of the original acti vator potency Chargaff Bancroft & Stanley Brown [78] prepared platelets from horse blood and isolated their various lipids A phos phatide fraction was found to contain a potent activator They con cluded: present methods for the separation of phosphatides are still much too crude to permit the isolation of compounds in a state even approaching purity It may very well be that a cephalin contain ing highly unsaturated fatty acids and therefore more soluble in ethyl alcohol is the real activator of blood clotting ' As in their later work on lung thromboplastin Chargaff et al [78] also noted that the 'defatted' material after platelet extraction was clot inhibitory In 1945 Chargaff [77] subjected normal human oxalated plasma to high speed (150 min at 20 000 r p m) centrifugation and lengthened the recalcification clotting times 3x or more In the following year fur ther studies [81] added information that the 'pellet' sediment from such centrifugation had thromboplastic properties similar to those of the 'macromolecular lipoprotein similarly obtained from lung ex tracts Flynn & Standley [167] reported to the Macy Conferences (1949) that their confirmatory observations also included microscopic examination of stained films which clearly sl owed that the pellet obtained by high speed centrifugation of human plasma is composed of platelets and occasionally erythrocytes Chargaff was mistaken then in identifying a 'plasma thromboplastin ' and was really just con firming Brinkhous' (1939) observations of the effects of platelet re moval on plasma clotting

In 1935 the present author knowing only of a short note of Hauro witz & Sladek [211] 1928 containing lipid analyses of platelets vis ited the Research Laboratory of the Children's Fund Detroit Michigan where a group of workers under Dr I G Macy were performing some excellent lipid (and other) fractional analyses of erythrocytes After preliminary work on some horse platelets prepared for the author by the Mulford laboratories the Michigan group completed a study on the lipid distribution of human platelets in health and disease [117] shortly after the appearance of Chargaff s work Sixty eight percent of the total platelet phospholipid analyzed as cephalin and it was com puted that if this were all liberated it could furnish 100 ml of blood with some 5 10 mg of cephalin This could be very significant in the light of the quantitative clotting data of Spagnol [447] and of Ferguson [129]

d) Platelets and the activation of prothrombin Eagle's [110] data were as follows:

(1) platelets (like cephalin) can participate in the activation of prothrombin (cf [328]) to thrombin Since Eagle's prothrombin acti vated slowly (about one hour) with $CaCl_2$ alone he concluded that platelets and cephalin accelerate coagulation by increasing the rate of

with Dr Douglas and Professor Macfarlane she showed that three components namely (1) platelets (2) $Al(OH)_3$-treated plasma (AHF factor V) and (3) aged serum (PTC factor ?VIII) in the presence of calcium interacted to generate this thromboplastin Samples of the above mixture removed from the pre incubate at minute intervals are added to platelet poor plasma (prothrombin fibrinogen etc) and observed to give progressively shorter clotting times through a period which is noted before they get longer again This is the thromboplastin generation test of Biggs and Douglas [48] The final clot timing resembles in principle the Quick prothrombin time test (p 57) but the preincubated mixture described above is substituted for the usual tissue (brain) thromboplastin

The Oxford group and investigators from other laboratories have applied the thromboplastin generation test system to the study of very many problems among which we may list the following

1) testing of platelet functions in blood clotting The platelet suspension may be tested (a) before and (b) after activation by plasma factors [225] (see pp 27 29) in normal subjects in thrombocytopenias [49] and in cases with functional platelet disorders (thrombasthenias or thrombocytopathias) [1 445]
2) substances able to substitute for platelets in the thromboplastin generation test include (a) a weak tissue thromboplastin [38] (b) cephalin [354] (c) chylomicra suspensions [406]
3) diagnosis of hemophilia and related disorders [313 378]
    (a) when the patient's $Al(OH)_3$ plasma gives a poor test the other reagents all being normal the result is presumptive evidence for hemophilia Ordinarily AHF persists in fresh $Al(OH)_3$ plasma along with AcG (factor V) whereas PTC (Christmas factor) prothrombin proconvertin (factor VII) and probably factor X are removed by the adsorption
    (b) when the patient's aged (overnight) serum gives a poor test the other reagents being normal the result suggests PTC deficiency (Christmas disease) PTC ordinarily survives in the serum along with factor VII whereas AHF and factor V are gone as is prothrombin (consumed) and thrombin (removed by the natural antithrombin)
4) certain circulating anticoagulants interfere with the test (Hougie & Fearnley [227] Hougie [226]) The present author and colleagues [152] have recently proved that these inhibitors are specifically anti AHF and anti PTC respectively We prefer our specific tests based upon the prothrombin consumption technique (p 46) because they make the distinction between the two inhibitors The thromboplastin generation test is unable to do this In other respects however it may be more sensitive than prothrombin consumption test methods A careful comparative study of the two methods has not been made and there is as yet no true yardstick for evaluating the validity of quantitative data of these and also other (e g [282]) methods
5) the question of intermediates;
    (a) Biggs Douglas & Macfarlane [50] found evidence for participation of factor V (AcG) and factor ?VII (proconvertin) in

used the two stage prothrombin method to assay residual prothrombin in serum after various intervals and thus determine 'prothrombin conversion rate ' This was much reduced in hemophilic plasma compared with normal It could be brought to normal however by adding lung thromboplastin In the careful preparation of the plasmas immediately centrifuging the citrated blood at low temperature the prothrombin conversion was found to be slower than in an earlier experiment Conversely when hemophilic blood was kept in citrate for 24 hours before removing the plasma from the cells the subsequent test showed great improvement in the prothrombin conversion as well as in recalcification clotting times

Eight years later with the advent of the silicone technique [251] Brinkhous [64] repeated efforts to obtain a platelet poor plasma and clearly showed that in normal plasma recalcification clotting times and 'prothrombin consumption (or 'utilization') depended on the numbers of residual platelets A platelet poor plasma became 'quasi hemophilic in these respects but normal platelet poor plasma corrected the defect when added to the hemophilic This was not so however when the hemophilic plasma was also 'platelet free ' He concluded in hemophilia there is a plasma factor required for platelet utilization

In further pursuit of the prothrombin utilization problem Brinkhous and colleagues [281] compared data obtained by the two stage test with the Quick one stage prothrombin method in both dogs and human each being (1) normal (2) hemophilic (3) platelet poor (by centrifugation) (4) blood clotting in silicone While both methods revealed the defective prothrombin consumption in (2) (3) and (4) the one stage values were not only higher but ran much above the original (0 time) value even in the normal This would appear to be due to the evolution and persistence of the recently recognized serum factor (convertin see p 26) which accelerates thrombin formation Evidently the two stage test is the more reliable measure of the prothrombin

Quick [385] in 1947 also approached the prothrombin consumption problem via hemophilia and modified his one stage prothrombin test (see p 57) by addition of fibrinogen later substituting $Ca_3P_2O_8$ a sorbed plasma [386 387] in order to apply it to (1 hr) serum (cf Soulier [444]) Like Brinkhous Dr Quick and his colleagues showed deficient prothrombin utilization in hemophilia and in platelet poor (centrifuged) plasma [391] They also studied the prothrombin consumption defect in (a) cases of thrombocytopenia [391 392] (b) a case of labile factor deficiency [390] and (c) after dicumarol [388] In one thrombocytopenic splenectomy raised the platelet count and restored the prothrombin consumption [392 388] Stefanini & Crosby [45_] have extended these observations

f) Platelets and thromboplastin generation, intermediates A new avenue of exploration of the blood clotting field particularly in relation to clinical bleeding disorders was opened up recently by the work of the Oxford University pathologists in the laboratory of Professor R C Macfarlane In 1952 Dr Rosemary Biggs [47] observed the development of a plasma thromboplastin in the platelet rich recalcified plasma from a rare case of congenital prothrombin deficiency Working

of Product I and the complete thromboplastin The last named is moderately stable in simple preparations but loses activity in serum or when citrated or oxalated The participation of factor V and factor VII ? is presumed in the formation of Product I and hence of complete thromboplastin but details of these relationships need further investigation

(c) Hörder has studied the defect in thromboplastic generation in a case with an alleged specific inhibitor of factor V [223] and has data on the influence of factor V upon plasma thromboplastin and hence thrombin formation [224]

Discussion on intermediates It appears to the present author that present methods are yielding conclusions which are suggestive rather than definitive The reagents used in the cited experiments are crude materials for the most part and this raises some questions In Macfarlane & Biggs [314] experiments for instance it was concluded that factors V and VII increased the potency of tissue thromboplastin What if any is the evidence that the potencies of factor VII and factor V are not being increased? Serum contains much proconvertin as well as convertin and pre incubation with calcium and thromboplastin can activate the former to the latter and abolish the lag phase in the conversion of prothrombin (factor VII free) to thrombin [295] Similarly a very minute trace of thrombin can activate proaccelerin to accelerin with similar abolition of the lag phase in purified thrombin forming systems [290] Can the English workers state the complete absence of traces of thrombin in their test system? These are debatable matters and the important point is that the experimental test systems leave the results open to divergent interpretations The final answers must await more definitive experiments It would seem that the distinction between the thromboplastin forming and the thrombin forming reactions is not yet wholly established

Conclusion Despite a diversity of approaches and viewpoints modern investigators in this field are providing evidence of similar basic concepts and of a common goal of inquiry It would now seem to be established that the many factors which participate in the thrombin forming reaction do so by a series of complex and inter related reactions in which the formation of certain intermediates would seem to be a logical necessity Many but not all of the data can be interpreted in terms of thromboplastin generation

g) Platelet factors related to clotting, etc At an April 1955 Conference of the National Research Council s Coagulation Committee meeting in Washington D C W H Seegers [426 104] listed eleven experimentally demonstrable actions of platelets or platelet fractions in the blood clotting system We quote his list verbatim

1 Platelet factor 1 (Ac globulin like property)
2 Platelet factor 2 (Thrombin fibrinogen interaction)
3 Platelet factor 3 (Prothrombin activation with plasma co factor(s))
4 Platelet factor 4 (Antiheparin)
5 Clottable factor
6 Antifibrinolysin activity
7 Antithromboplastin activity
8 Clot retraction
9 Vasoconstriction (serotonin)
10 Snake venom factor
11 Ac globulin stabilization

thromboplastin generation from platelets Macfarlane & Biggs [314] also showed an increasing potency when an already active brain ex tract was preincubated with factors V and VII (cf [320 319 318]) This raises considerable question as to terminology and Macfarlane et al suggest that the term 'thromboplastin' should really apply to the product of the preliminary reactions which with calcium participates directly in the conversion of prothrombin to thrombin Since Howell (p 9) although not strictly in that author's sense the term thrombo plastin has been widely used to designate any tissue extract used in activating prothrombin Brain thromboplastin for instance is the key reagent in the Quick prothrombin time test (p 57) Brinkhous and colleagues (p 46) distinguish between tissue thromboplastin as a 'complete' thromboplastin and cephalin or platelets as 'incomplete' or 'partial' thromboplastins They apply these ideas in their two stage prothrombin consumption test (p 46) and partial thromboplastin AHF assay [65 66]

Actually the historical term (p 8) for the postulated activator with calcium of the prothrombin conversion was 'thrombokinase Hence there is much merit in Milstone's [339] suggestion to re define this term for the present purposes Milstone's work while confined to experimental test systems essentially anticipated much of the basic concepts advanced by the more recent English investigators Other American researchers notably F D Mann and associates [320 318] Flynn & Coon [165 166] have pursued the idea of intermediate com plexes in the thrombin forming reactions although not emphasizing quite the same viewpoint as the above The distinction between throm bin generation [312 377] and thromboplastin generation is by no means clearly established at this time however (see p 49)

(b) Hougie [225] has studied the shortening of the recalcifica tion time of platelet rich plasmas from cases with (1) circulating anti coagulant (2) hemophilia (3) Christmas disease (4) 'dindevan' (phenyl indanedione related in action to dicumarol and tromexan) and (5) nor mal bloods He concludes that there is another important intermediate at a later stage in these complex reactions The following summary of these ideas was kindly supplied by Dr Hougie in a personal communi cation According to Bergsagel & Hougie [40] AHF and PTC undergo a reaction with calcium to form an intermediate product (Product I) Product I reacts with platelets causing them first to agglutinate and then to lyse (Bergsagel) resulting in a sedimentable thromboplastin complex believed to be a complete thromboplastin This (a) differs from tissue (e g brain) thromboplastin (b) in that the one stage pro thrombin time of stored and dindevan plasma considered to be defi cient in both factor V (proaccelerin) and factor VII (proconvertin) respectively is normal with (a) but delayed with (b) This is true even when (a) is washed and re sedimented and used in high dilution The complete thromboplastin loses its activity on heating for 10 minutes at 56°C However after heating for 5 minutes to 100°C and re treating with Product I the activity is restored and may again be recovered in the (ultra)centrifugal sediment The heat stable component is thought to be cephalin and the heat labile component to be Product I Prod uct I loses its activity after decalcification by Amberlite (ion exchange resin) and therefore calcium is believed to be an essential component

for the possibility of loss due to lability of the AcG like factor we in clined to the view that it might merely represent some of the plasma AcG adsorbed onto the platelets This view is strongly supported in a recent study by Hjort et al [222]

7) A factor contributing to AcG instability was in cidentally noted by Ware Fahey & Seegers [490]

8) A factor contributing to thrombin stability was incidentally noted by Travis & Ferguson [476]

9) A fibrinoplastic factor (the present author's term see p 17) aiding the thrombin fibrinogen reaction was claimed by Ware Fahey & Seegers [490] and also reported by van Creveld & Paulssen [481] and by Jurgens [261] Travis & Ferguson [476] could not con firm this however Platelet Factor 2 (Seegers) denotes this alleged activity

10) A clottable factor [490] refers to the clot like' reaction of platelets to the addition of thrombin The author s (unpublished) dark field observations indicate this to be a variant of the viscous metamorphosis' (p 35) of platelets and not any true clotting phenome non

11) Platelet vasoconstrictor or serotonin has also been considered separately (p 39) Also ref Correll et al [94] and Humphrey & R Jacques [243] Magalini & Stefanini [315] could not confirm Fenichel & Seegers claim [121] for a clot retraction effect of serotonin

12) An antifibrinolytic factor was noted by Seegers col leagues Johnson & Schneider [257] and confirmed by Stefanini [450] Also see p 119

Conclusion This is obviously a rich field for further investigation to determine the exact identity of the factors involved and the way in which platelets can participate in the phenomena mentioned

h) Erkelens thesis A recent comprehensive thesis [118] of the role of platelets in the coagulation process by A D Erkelens (Rot terdam) has appeared in Holland Its chief conclusion is that plasma changes precede and are probably responsible for platelet breakdown In platelet free plasma coagulation can be induced by kaolin and other particulate matter As a result of the secondary structural changes of the thrombocytes after the formation of the first fibrin a thermo stable thrombocyte factor is liberated stimulating the formation of thromboplastin Ther fore an accelerating and finishing function in the proces of blood coagulation is assigned to the thrombocytes but no initiating function

Stefanini [449] includes some detail as to platelet factors in his 1953 review Ackroyd's recent review [1] cites "platelet factors 1 4 and these are so titled and discussed in much of the current work in this field We shall not attempt any comprehensive review of all the literature on this topic but will present our own attempt to order the experimental facts in some logical order with a few selected references to each category

1) Platelet thromboplastin' (preformed) The best evidence for this is Chargaff's [78] preparation of 'platelet thromboplastin' as a lipoprotein centrifugate From this he recovered 'platelet thromboplastic phospholipid' (? cephalin) in lipoidal extracts That this is a weak and 'incomplete' thromboplastin is evident from the data of Quick [387] Ware Fahey & Seegers [490] Travis & Ferguson [476] and others

2) Platelet component (platelet Factor 3) in generation of plasma thromboplastin Lenggenhager [287] used the term 'thrombokinin' and Quick [387] the term 'thromboplastinogenase' in looking at this from one point of view namely as if the platelet component were the activator of the plasma components (see AHF = 'cofactor I' and PTC = 'cofactor II ' etc p 28) It is really semantic (or tautological) but most other workers prefer to present the problem from the opposite point of view namely as if the plasma cofactors activate the platelet precursor of a thromboplastin Bergsagel and Hougie's views as to probable 'intermediates' in these reactions have been stated (p 48) Alkjaersig Abe & Seegers [12] have attempted to purify platelet factor 3 and guardedly state that it is unlikely to be a protein In their best preparations it has an N P ratio of 8 4 1 No discussion of the reactions which these authors claim for platelet factor 3' can be adequate without going into the many details of Seegers' current theories which we do not propose to review in the present dissertation

3) Snake venom factor according to Lee Johnson & Seegers [285] refers to some platelet factor which acting in conjunction with Russell's viper venom (itself a 'thromboplastin ' with certain restrictions of potency [398 436 500]) is able to activate purified prothrombin rapidly in experimental systems Interrelations between venoms and phospholipids is a topic with a considerable literature and we shall not explore this any further See SUPPLEMENT

4) Antiheparin action (platelet Factor 4) claimed by van Creveld and Paulssen [481] and recovered in a separate fraction by Deutsch et al [104] Other recent references [261 490]

5) *Antithromboplastic action* : *ref Jurgens* [261] *thermo* labile factor sedimentable in the ultracentrifuge

6) Platelet accelerator (platelet Factor 1) was discovered by Ware Fahey & Seegers [490] and probably accounted for some of the data of Quick [385] and of Mann Hurn & Magath [320] Travis & Ferguson [476] found only traces in well washed platelets Allowing

PART II

EXPERIMENTAL

A GENERAL PURPOSE

The following experiments seek further knowledge concerning the role of lipids and platelets in the blood clotting mechanisms Blood its platelets plasma and plasma fractions and a number of tissue components are analyzed with regard to the various factors which they contain and which can be demonstrated to play a part in the interactions involved in these mechanisms The significance of the platelets and some of the other factors is considered in relation to hemorrhagic disease In vitro test systems are devised to demonstrate the modes of action of the various factors and agents studied many experiments being performed by this means

B GENERAL METHODS

The materials used in these investigations are for the most part derived from mammalian blood namely dogs humans and beef cattle

1 BLOOD COLLECTION

1a) Dog Blood samples of some 10 50 ml (or more) may often be obtained from the jugular vein of the unanesthetized dog with minimal discomfort using a syringe and needle technique as in the case of humans (see 1b) For large amounts of blood it is desirable to sacrifice the animal under intraperitoneal sodium pentobarbital (nembutal) or similar anesthesia The carotid artery is cleanly exposed and cannulated with a siliconed glass cannula or polyethylene tubing Controlling the vessel with a soft rubber tipped bulldog clamp and avoiding tissue contact e g by discarding the first few ml of blood collection is made into anticoagulant or otherwise as needed using glass (pyrex) Erlenmeyer flasks or siliconed bottles Oxalation or citration requires 9 volumes of blood to 1 of 0 1 molar sodium oxalate or trisodium citrate Sequestrene Na_2 (EDTA or 'Versene) is reserved for special collections e g for platelet studies the routine being to receive 5 ml of blood into a siliconed 1 oz bottle containing 50 mg of the powdered chelating agent cf p 276 of ref [453]

1b) Human An extensive testing routine [172] may be performed on 20 40 ml of blood obtained aseptically from the antecubital vein using a siliconed syringe with a sharp new short bevel No 20 needle Arquad 2 C (2 percent solution) coating of needles (p 283 of ref [453]) has little additional advantage The blood is immediately transferred into anticoagulants as in 1a and 4 5 ml of blood is also clotted in a small (Wassermann type) tube held in the water bath at 37°C This is

Other adsorbents used in some of our earlier work include $Mg(OH)_2$ [176 442] which is too alkaline in our opinion; type C^{α} $Al(OH)_3$ gel [381 52] $Ca_3(PO_4)_2$ [56 387]

4 FIBRINOGEN

For purposes of the experiments described in this thesis the most satisfactory fibrinogen is prepared from dog oxalated plasma which has been adsorbed with $BaSO_4$ as in 3 The fibrinogen is salted out at ¼ sat $(NH_4)_2SO_4$ the centrifuged precipitate dissolved in saline and dialyzed against saline containing 0 005M trisodium citrate Some purer fibrinogens will be described in certain experi ments

5 PROTHROMBIN PREPARATIONS

5a) Howell type [71] This was much used in our earlier studies before the advent of the modern types of preparation It was made from Berkefeld filtered citrated dog plasma (see Experiment 22 p 91) by means of acetone precipitation (after heat defibrination) collecting the precipitate on filter papers washing with ether and drying For details of preparation of solutions see p 91

5b) Seegers type These are the purest prothrombin preparations to date They are secured by adsorption of citrated plasma (bovine) with $Mg(OH)_2$ and subsequent elution with CO_2 under pressure see p 15 [435 431] We have had some personal experience with this type of preparation but have used many that were kindly prepared for us by Dr Seegers ref [156]

5c) Eluate type In recent work including many of the experiments in this thesis we have obtained a prothrombin rich solution from dog human or bovine oxalated plasma by adsorbing with $BaSO_4$ (see 3 above) and subsequently eluting the twice washed (with distilled water) sediment with 1/3 volume 0 2M trisodium citrate see p 60 Analyses on these eluates will be described in several of the experiments

C REAGENTS

1 COMMERCIALLY AVAILABLE PRODUCTS

Chemicals such as various salts solvents etc usually require choice of the best 'analytical' grade and careful and accurate prepara tion of solutions

Sp cial materials which are currently available include

1) Armour & Co 's bovine fibrinogen plasma Fraction I ' lyophile dried the preparative methods being based on the Harvard Plasma Fractionation Commission s cold ethanol technique [114] We routinely remove traces of prothrombin etc by $BaSO_4$ adsorption 1 g/100 ml of 1% solution of B F is sufficient

2) Human fibrinogen prepared by this method and available for re stricted distribution by the American National Red Cross

3) Incubation mixture for performance of the two stage prothrom bin assay (according to Ware & Seegers [495]) obtainable from the Difco Corporation (courtesy of Dr C W Christensen)

later centrifuged in order to recover the serum As a routine for prothrombin consumption determination 0 5 ml of 0 1M sodium oxa late is added after exactly 1 hr immediately before centrifuging

$1b_1$) Special blood donations, e g normal hemophilic PTC deficient etc are secured through cooperation of the Blood Bank and the citrated or oxalated plasmas are reserved or fractionated for par ticular test purposes Frozen storage at 20°C ('Deep Freeze') is a *routine*

1c) Beef Ox blood is obtainable at a cooperative slaughterhouse where it is possible to arrange for suitable large scale collections With suspension of the cleansed animal and skill on the part of the butcher there is such a rapid flow of blood that very little objection can be raised to its slight contact with the wounded tissues Several gallons of blood may be secured in thoroughly clean galvanized pails containing the appropriate amount of anticoagulant The mixture is quickly transferred to large pyrex bottles kept chilled in buckets of ice for transport to the processing laboratory

2 PREPARATION OF SERUM PLASMAS PLATELETS

2a) Natural serum Whole blood is clotted in tubes (or bottles) held in the water bath at 37°C for 1 hr The serum is then separated from the retracting clot by suitable centrifugation

Oxalated serum This is described in Ib above

2b) Plasmas e g oxalated or citrated are secured by centrifuging the blood containing the anticoagulant in glass lusteroid or poly ethylene tubes The refrigerated (4°C) 'International' centrifuge is recommended and it is best to perform the operation in two stages namely 15 min at 1500 r p m and recentrifugation of the supernatant cell contaminated plasma at 3000 r p m for ½ - 1 hr

2c) Platelet rich plasma Versene treated blood p 53 is trans *ferred to siliconed tubes and centrifuged at 800 r p m for 20 min* The supernatant plasma is rich in platelets and if only the upper por tion is removed there will be a reduced contamination with leucocytes and erythrocytes

2d) Platelet poor plasma, The top 2/3 only of the above (2c) platelet rich plasma is carefully removed and measured with a sili coned pipette The measured volume is transferred directly into the 6 ml capacity siliconed tubes of the 'multispeed' attachment of the re frigerated 'International' centrifuge and spun for at least 45 minutes at 20 000 r p m The clear supernatant avoiding the lowermost por tion is all but free from platelets

2e) Platelets The sedimented platelets from 2d are washed 6x with 0 85% NaCl (saline) containing 0 2% Triton (WR 1339 Rohm and Haas ref [453]) and finally resuspended in 1/10 the volume of the original platelet rich plasma

3 $BaSO_4$ ADSORPTION ETC [98 463 410 194]

Ten minutes adsorption of plasma with pure $BaSO_4$ e g Merck's 'reagent ' at 100 mg per ml removes prothrombin proconvertin PTC etc leaving most of the fibrinogen AcG AHG etc

b) The prothrombin time determination patterned after the original one stage test of Quick et al [393] is performed as follows To 0 1 ml plasma is added 0 2 ml of Ca tpln (equal volumes of 0 02M $CaCl_2$ and tissue thromboplastin e g 'Soluplastin ' p 56) and the appearance of fibrin timed with a stopwatch at 37°C

c) Thrombin forming factors, assayed by specific ONE STAGE methods By modifying the above technique we (and others) have overcome the non specificity of the original test and introduced specific bioassays for (1) prothrombin (2) (pro)accelerin and (3) (pro)convertin

The three methods differ in the choice of substrates (see below) They all follow the common procedure of adding 0 1 ml of 1:10 (or suitable) dil plasma (or serum or other agent) to 0 1 ml of the appropriate substrate in a 13 mm diam serological tube in a 37°C waterbath then adding 0 2 ml Ca tpln and timing the clot (film at edge) with a stopwatch

Ca tpln equal vols 0 02M $CaCl_2$ and Soluplastin (p 56) or other suitable tissue thromboplastin A normal designated 100 per cent ' is the mean of a statistically significant number of assays on plasmas from healthy normal adult human subjects From (a) such a normal or better (b) a hyperreactive pool from a number of cases at or near the upper limit of normal variations a series of saline dilutions (preferred to other diluents) is made and the data are plotted as a standard reference curve Values up to about 120 130 percent of the statistical norm are included in the curve obtained by method (b) and most plasma unknowns will fall on this reference curve In the case of reagent testing variable dilutions are necessary

To overcome day to day variations of reagents and test conditions it is recommended that a control of the day' be selected from available personnel whose assay point on the standard curve has been repeatedly established This test will then orient the standard curve for the particular set of tests as shown in the following example

Patient 'X' gives a one stage clotting time (say for prothrombin) corresponding to 63 percent on the standard reference curve The day's control known to be 90 percent on the standard curve today gives a clotting time which reads at 81 percent Hence the corrected UNITAGE of the unknown 'X' is 63 x (90/81) - 70 expressed as percentage' of the standard normal In the present study reciprocal prothrombin times are also expressed percentagewise in terms of a standard normal 'activity Figures 9 10 11 12 show correlation graphs of four methods namely prothrombin' time Method I (specific one stage prothrombin assay) Method II (orthodox two stage prothrombin assay) Method III (eluate assay method for prothrombin) They represent over 300 tests on plasmas from human cases both normal and with clinical variations to the limits of the test methods

The excellent correlation (ρ - 0 95) of prothrombin data obtained by the specific 1 stage (Method I) and the orthodox 2 stage (Method II see below) is graphically illustrated in Figure 10 Incidentally as shown in Figure 9 there is a very much poorer correlation (ρ - 5) of Method II and the unmodified prothrombin time test due to the now recognized fact that the Quick test is influenced by variables other than prothrombin All three specific methods show excellent correlation

d) Method I, Specific 1 stage prothrombin assay Our modification of the method of Owren & Aas [365] uses as substrate a $BaSO_4$

4) 'Simplastin' (Ca-thromboplastin) is available from the Warner Chilcott Co and is reliable for prothrombin time tests

5) 'Soluplastin ' Schieffelin and Co (courtesy of Dr E W Blanchard) is the commercial thromboplastin used for most of the tests in the present study

6) Bovine thrombin Upjohn & Co (courtesy of Dr J T Correll) is preferred

7) 'Thrombin topical' (bovine) Parke Davis & Co (courtesy of Dr E A Sharp) also is good but is often unduly contaminated with fibrinolysin

Many Individual investigators have provided us with samples of their materials which will be acknowledged in appropriate sections Our own procedures and preparations will also be described and critically discussed in connection with their use in various experiments

2 TESTING OF REAGENTS

While the special qualitative and quantitative characterization of reagents will entail testings which are part of the individual experiments there are some general test procedures which are appropriately reviewed at this time The following selected techniques represent the fruits of many years of experience They embody experimental criteria based upon many lines of progress by a host of modern workers in this field However many of them were not available in the earlier periods of our study when some factors much less techniques were still unknown Hence these test methods are presented as recommendations in the light of which old experiments may be reviewed and re evaluated and sometimes profitably repeated perhaps with modifications suggested by the newer ideas

a) Fibrinogen assay (I) Gravimetric The common clinical routine [453 473] is quite useful for approximate determinations on isolated fibrinogen solutions Add 0 5 ml bovine thrombin (500 units/ml) to 0 5 ml solution (e g plasma) diluted with 5 ml imidazole buffered saline Imidazole buffer [331] of pH 7 3 is made by dissolving 1 72 g imidazole (Edcan Labs) in 90 ml 0 1N HCl and diluting with distilled water to 100 ml One vol imid buff is added to 9 vols saline (0 85% NaCl) The fibrin clot is carefully and completely wound off on a tared glass rod washed with saline followed by distilled water and dried to constant weight i e overnight at 85°C and weighed

(II) Colorimetric [348] Our [60] preferred method for highly accurate assays of purified fibrinogen is to recover the thrombin fibrin obtained as in (I) on a mounted dissecting needle washing digesting with a modified Mehl's biuret reagent then diluting and reading the color intensity with the Klett Summerson photoelectric colorimeter using No 540 filter The instrument is previously graduated against the above gravimetric (dry weight) and macro Kjeldahl (fibrin N) determinations

(III) Turbidimetric In 1942 the author [135] used the Evelyn photoelectric colorimeter with No 540 filter as a turbidimeter Supplementary to other studies it was noted that the turbidity of clots formed by adding thrombin to a 1 10 dil plasma could accurately measure the addition of fibrinogen in amounts as small as 7 5 mg/100 ml A recent micro method [512] is based on these principles

f) Specific 1 stage proconvertin a say uses as substrate bovine plasma twice filtered through special '20%' asbesto (Seitz type) pads obtained from the St Gallen Co Switzerland according to the original recommendations of Owren & Aas [365] This filtration is designed to remove nearly all the proconvertin but to leave a sufficient amount of prothrombin Our tests usually reveal about 60% of the original prothrombin and a control prothrombin time test (with 0 1 ml saline instead of test plasma) of 120 sec or longer A new recommendation [2] to employ a wood charcoal 'L 50' (Springfield Facing Co Williamsett Mass) may be noted We have at times supplemented our substrate with proconvertin free bovine prothrombin prepared by the method described for human plasma fractionation [295] Fibrinogen and other factors are satisfactory in this substrate but like the prothrombin substrate (p 58) it may lose AcG and require supplementation of this in the test plasma dilution

g) Method II The orthodox TWO STAGE prothrombin a say The original two stage assay [496] has been modified [495] Ferguson et al [153] have recently made a critical restudy of the two stage prothrombin assay method both as to technical considerations and in application to the investigation of bleeding disorders Our improved' test adds 1% bovine proconvertin (see above) as well as AcG (the 'modified' method) This is actually essential only in very rare instances when testing plasmas etc which are extremely low in proconvertin e g some congenital hypoproconvertinemics Briefly detailed the method requires four steps namely

Step 1. D fibrination 0 5 ml plasma + 0 4 ml saline + 0 1 ml thrombin (bovine Upjohn's 20u/ml) remove clot with glass rod over 15 minutes

Step 2. Dilution Appropriate (e g 1 30) dilution of 0 1 ml(1) with 1 100 (saline dil) AcG + proconvertin

Step 3. Activation 0 6 ml (2) + 1 8 ml incubation mix consisting of 11 5 saline 1 imidazole buffer 4 purified (Ca free) acacia 15% (Wills Corp) 0 6 Soluplastin (Schieffelin & Co) 0 9 $CaCl_2$ (0 1M)

Step 4. Clot timing 0 4 ml samples of (3) removed at 1 min intervals are tested with 0 1 ml fibrinogen at 28°C (25° 30°) noting the minimum reached This should be in the 13 17 sec range

Our best standard fibrinogen is simply prepared from oxalated dog plasma $BaSO_4$ adsorbed as usual (see p 55) precipitated at 1/4 saturation $(NH_4)_2SO_4$ redissolved in 1/3 orig vol saline and dialyzed against citrated saline (0 005M sodium citrate) Frozen stored (20°C) in 10 ml lots it keeps well for many months with a remarkably constant reactivity (see p 62) Occasionally a small amount of sediment appears on first thawing but this can be centrifuged off and the bulk of solution remains clear thereafter even through repeated freezings and thawings

Computation of unitage E g if a 30x dil is used in stage (2) and the minimal clotting time (reached say in 3 or 4 min of step (3) incubation) is 14 sec and hence the correction factor from Ware & Seegers' tables [495 256] is 1 1 1 1 x 5/4 x 4/1 x 30/1 x 2/1 (i e respective dilutions at steps 4 3 2 1) 330 units/ml

h) Method III. $BaSO_4$ citrate ELUATE techniques a new two stage method developed in the author s laboratory METHOD 3 ml

adsorbed beef plasma fortified with 1/10 vol of a bovine serum proconvertin preparation This proconvertin is prepared from ox serum by the same method essentially as we [295] used in the case of human plasma The serum was obtained by recalcifying oxalated beef plasma and centrifuging after standing for 18 hrs at 4°C Interestingly this reagent although prepared from serum is mainly unchanged proconvertin We deduce this by observing that it does not abolish the 'lag phase' in prothrombin conversion as compared with convertin (proconvertin pre incubated with $CaCl_2$ and thromboplastin) which does

The prothrombin substrate is designed to contain the normal plasma amounts of fibrinogen proaccelerin and other possible factors not adsorbed on the $BaSO_4$ The $BaSO_4$ has removed the prothrombin (hence its suitability as a test substrate) the proconvertin (hence the re addition of this factor) PTC and possibly other materials which are not believed to play any role in the conversion of prothrombin to thrombin in the presence of Ca tpln Frozen stored in 5 ml lots at 20°C this prothrombin substrate remains serviceable for many months Occasionally it loses significant amounts of AcG although this is far more stable in bovine than in human plasma In such case the substrate should be assayed for prothrombin by the two stage method and if this is satisfactory the substrate can be restored to usefulness merely by using a suitable dilution of AcG instead of saline in the (1 10) dilution of the test 'unknown '

Serum AcG (accelerin) as used for this and other (see two stage etc) purposes is prepared according to the directions of Ware & Seegers [495] i e ox serum aged several hours is adsorbed with 4g/100 ml $BaCO_3$ $BaSO_4$ can be substituted for the carbonate

e) Specific 1 stage proaccelerin assay uses as substrate 'aged' human oxalate plasma as originally recommended by Quick & Stefanini [395] For some time (e g [297]) we recommended $BaSO_4$ adsorption (the usual 100 mg/ml) of the unknown plasma before the routine 1 10 dilution prior to adding to substrate etc However any additional prothrombin which would be carried over in the diluted unknown to the normally prothrombin rich substrate can be shown to have a negligible effect particularly when the reference standards are handled in the same way The easiest method therefore is to employ untreated 1 10 unknown' plasma etc as in the other specific 1-stage methods In reagent (e g AcC) assays there is again no need for pre-treatment unless the material contains a large amount of prothrombin

'Aging' of the substrate plasma is best accomplished by freezing and thawing (which always reduces the labile proaccelerin) and storage in the ice box at 4°C for one or two weeks (or longer) until the regular prothrombin time test (0 1 ml plasma + 0 2 ml Ca tpln) at 37°C gives clotting times in excess of 60 seconds

Aged plasma should contain normal amounts of fibrinogen prothrombin proconvertin etc Frozen stored it is serviceable for many months and if a small amount of sediment appears on thawing this can be removed by centrifugation and the residual supernatant found to be little affected in most instances A very fresh normal plasma must be used for the standard of reference and for the daily control We have found it best to use a single well known 'normal' for both

Standards for the accelerator factor assays are a series of activation curves obtained with several dilutions of a comparable normal whether (k_1) plasma or (k_2) platelet suspensions after the manner of the Ware & Seegers two stage AcG assay [493]

ℓ_1) Thromboplastic factor assay by the eluate method: Using essentially the same procedure but substituting for the regular thromboplastin ('Soluplastin) any material containing a thromboplastic factor can be assayed e g tissue thromboplastins (ℓ_1)

ℓ_2) Platelet thromboplastic factor is routinely assayed in our laboratories by this technique It involves platelet preparation (as above) and normal platelet standards with the reference series of dilution curves (activation curves)

Cephalin and a variety of lipids and tissue thromboplastic extracts will be studied by this method in experiments to follow

D INHIBITOR MECHANISMS

These will barely be mentioned in the present thesis but it must be noted that certain inhibitors of the thromboplastic mechanisms or perhaps of other components in the prothrombin conversion reaction can be demonstrated by delay in thrombin formation (compared with inhibitor free controls) in such test systems as these of the eluate method Other prothrombin activation tests (showing inhibitory phenomena) will also be used in the present investigation

1 Heparin (with its cofactor) is an important experimental addition in some of the present test systems

Testing for heparin in blood or plasma is usually done by (a) *whole blood clotting time tests* or (b) *protamine titration* (with which we prefer to use a plasma recalcification system) but details of these techniques will be omitted

2 Antithrombin tests

a Plasma thrombin clotting time test The clotting time (at 37°C) of a test plasma mixed with a standard solution of thrombin and compared with a similar experiment using a normal plasma is often suggestive evidence of some 'immediate (or rapidly acting) antithrombic factor An example is seen in the new born infant [172]

b Serum antithrombin refers to the 'progressive' loss of thrombic activity when a standard thrombin is incubated with serum (or other fibrinogen free material) and retested with fibrinogen at successive time intervals Saline may be substituted in the control

This test is performed with many of our test materials when we wish to demonstrate the presence or absence of antithrombin An example is seen in Table VII

Often however it is enough merely to retest a thrombin forming mixture at intervals to observe definite antithrombic action This is always seen for instance in the orthodox 2 stage prothrombin test and raises some theoretical questions as to the true end point In practice at the high dilutions tested this is usually ignored however

E STUDIES ON ARTIFICIAL CLOTTING SYSTEMS

1 TEST METHODS In general test systems are quantitative using

oxalated (not citrated) plasma is adsorbed 10 min with 300 mg $BaSO_4$ (Merck's) and the sediment recovered by centrifugation is washed with distilled water and then eluted for 10 min with 1 ml of 0 2M sod citrate *The eluate recovered by centrifugation contains essentially* all the prothrombin proconvertin and PTC of the original plasma It lacks fibrinogen proaccelerin (except for traces) AHF and most of the plasma inhibitors (e g antithrombin) usually

i) Eluate assay (Method III) for prothrombin, by new two stage, 0 1 ml eluate is incubated at 28°C (or approximate room temperature) with the following activator system 3 6 ml saline (0 85% NaCl) 0 5 ml (i e 1/10 vol) imidazole buffer (pH 7 3) 0 1 ml bovine procon vertin (see p 58) 0 1 ml AcG ($BaCO_3$ bovine serum see p 58)diluted 1 8 0 1 ml 'Soluplastin' (Schieffelin & Co 's thromboplastin) and finally 0 5 ml 0 15M $CaCl_2$ At 1 min intervals 0 2 ml samples are tested with 0 2 ml fibrinogen (Armour's bovine 'Fraction I ' 1% *treated with 1 g $BaSO_4$/100 ml) Clotting times recorded with a* stopwatch usually reach a minimum within 5 minutes and because of lack of inhibitors are typically stable or nearly so for many minutes thereafter

Minimum clotting times are converted into prothrombin 'percent age' of normal by reference to a standard curve of data obtained from dilutions of a normal pool of plasmas as described under the 1 stage assays A 'standard of the day' is also routinely used in the manner previously outlined (p 57) Illustration of the technique used to assay standard eluates is seen in Experiment 10 (p 75)

Correlation between Method III and the orthodox 2 stage (Method II) prothrombin assays for the same large group of (300) human cases *as studied in the other comparisons of prothrombin methods is shown* to be very satisfactory in Figure 12

j) A 'proconvertin index' by modification of the eluate method: By omitting the 0 1 ml of proconvertin from the activator system in the previous test and securing the first minute test without it a clotting time value is obtained which using the reference curve indicates the initial velocity of the prothrombin activation This is due chiefly to the proconvertin present in the plasma eluate By then adding the 0 1 ml proconvertin and continuing the usual incubation and testing the prothrombin activation is carried to completion The ratio of percent age units in the 1 min test divided by the final (e g 5 min) percentage is a 'proconvertin index ' which we have found to give a fair degree of correlation with the routine 1 stage specific proconvertin assay (p 59)

k 1) Eluate method of assaying (1) accelerator or (2) thrombo plastin A STANDARD eluate is made essentially by the method de scribed for human plasma but using bulk volumes of either (a) dog or (b) bovine oxalated plasma Using this as a substrate the prothrombin conversion may be studied at 1 min or other suitable intervals with the regular activator system modified by substitution for the cited (1) accelerator globulin or (2) Soluplastin ' of any other accelerator or thromboplastic material it is desired to study

k_1 k_2) The new two stage AcG assay can be used for example on (k_1) fresh plasma ($BaSO_4$ adsorption is necessary) or (k_2) platelet suspensions (fresh) to assay their 'accelerator' property See p 54 for method of preparing washed platelets

in this way that the National Institutes of Health standard is maintained

c) Standardization of fibrinogen. It is reasonable to standardize every batch of fibrinogen by repeated clotting tests with a standard thrombin as performed at N I H In our laboratories this is not attempted Either the particular batch of fibrinogen unstandardized is regarded as the 'standard' for a particular set of experiments relative c t values being acceptable in the absence of any evidence of fluctua tions Or when a particular fibrinogen is used for routine two stage prothrombin assays of plasma from normal subjects and human clin ical cases sufficient experience is secured with the fibrinogen to char acterize its reactivity using a statistical mean of cases with normal plasma prothrombin unitage [153] We have prepared many dog fi brinogen preparations (p 55) with which the two stage prothrombin assay gives close to 300 units/ml human plasma which is a very ac ceptable value

d) Clotting times and thrombin concentration Returning to the first method while this has no value in terms of any absolute unitage it nevertheless gives perfectly valid relative values if some refer ence series of thrombin dilution is used under the same experimental conditions The fundamental principle is that the shorter the clotting time under standard conditions the more the thrombin activity or rel ative concentration This is here put into practical use being validated as mentioned only for a given set of comparative experiments By ex perimentally obtaining the data and plotting graphically as a thrombin dilution curve ' e g Figure 5 the observed clotting times in any vari ation of the experiment may be read off as a 'percentage of the orig inal (strongest) thrombin Much valuable information does not even require exact quantitation in this way For instance a very long clotting time is clearly a matter of a very low thrombin concentration and two such tests may be far below the quantitative limits of the method and yet be significantly different by simple inspection of the data

4 THE INVERSE LAW

In the example given in Figure 5 (see p 18) quite by chance a very fair approximation to the 'inverse law' [141 162 20] was obtained In our experience the true variables responsible for the usual diverg ence of data from this 'law' have not been defined and we do not usu ally attempt to secure this exact type of linear reference curve A recent (unpublished) study of one possible variable however is illus trated in the following experiment

F EXPERIMENTS

I EXPERIMENTAL CONDITIONS MODIFYING THE THROMBIN FIBRINOGEN REACTION

The thrombin fibrinogen reaction has been discussed on pp 16 et seq in the introductory part of this thesis Among the data there re viewed are those of Boyles Ferguson & Muehlke [60] dealing with a number of experimental variables in the thrombin fibrinogen reaction The first two experiments of the present thesis add new data obtained

accurate measurement of amounts of reagents including use of volu metric (e g serological) pipettes and other glassware always kept scrupulously clean with a cleansing routine involving detergent (Dural H) sulfuric acid chromate solution distilled water and drying oven Timing of many procedures is facilitated by electric chronometers and mechanical stopwatches Clotting times are used as 'end points' in the great majority of experiments

2 CLOTTING TIME as discussed on p 18 is an empirical observa tional datum which becomes significant only under strict conditions which will be defined for the individual experiments

3 THE THROMBIN FIBRINOGEN REACTION

The key reaction in the physiological mechanism of blood clotting is the conversion of fibrinogen into fibrin through the agency of throm bin The first step in our investigation therefore must be an evalua tion of the time relationships of the thrombin fibrinogen reaction

a) Fibrinogen and clotting time Fibrinogen concentration over a considerable range [60] has remarkably little effect on clotting time (c t) Of much more significance is the reactivity of the particular fibrinogen solution meaning the particular clotting time it will show *with a given thrombin preparation under the defined test conditions* There is no real information to date upon which we can predict the reactivity of a fibrinogen It is also not exactly known what determines the potency of a particular thrombin preparation However any of the modern highly purified and stable thrombin preparations may be relied upon to give a definite clotting time reproducible within a small limit of experimental error in many repetitive tests conducted with a given good fibrinogen solution Under carefully controlled and standardized experimental conditions repeatedly using the same substrate fibrinogen comparative clotting times afford a valid though relative measure of thrombin potencies Actually two variants of method have both been widely used In the first the thrombin concentration is varied and the respective clotting times used as a measure of relative thrombin po tency An example is given in Figure 5 in the introductory section In the 'method II (p 59) the thrombic material is diluted to give a standard clotting time and relative dilutions are used as the measure of thrombic activity

b) Standardization of thrombin The National Institutes of Health (Bethesda Maryland) provides a thrombin standardized by this second method the original (revised) procedure being that of Smith Warner & Brinkhous [443] as modified by Ware & Seegers [495] The method was originally (1934) developed to assay prothrombin in terms of the thrombin unitage developed after maximal activation to thrombin The unit whether for thrombin or activated prothrombin is defined as that potency of the product which gives a 15 second clotting time of a particular standard fibrinogen under the stipulated experimental condi tions Difficulties in securing a standard fibrinogen are insuperable as previously mentioned but a practical solution has been to have many testings of a particular lot of thrombin and then to retest many times when the original fibrinogen needs replacement with a new batch It is

also have been removed in the isoelectric method of purification Ar guing for only a trace impurity is the observation that the $(NH_4)_2SO_4$ purification of Armour's fibrinogen gave a 97" clottable preparation which failed to obey the inverse law in the experiments of Boyles and the author [60] Ammonium sulfate fractionation does not eliminate the Lorand factor [439]

Experiment 2 (1956) Table III

Purpose In the light of the cited recent work [307 439] clearly re lating the Lorand 'serum factor' to calcium and the opacity rigidity and urea insolubility of fibrin clots is it possible to explain the anom alous action of calcium salts in a limited concentration range namely shortening the clotting times of thrombin fibrinogen mixtures on the basis of the reactions involving the serum factor?

Test Under constant volume and fixed thrombin and fibrinogen (I III) concentrations $CaCl_2$ was added to the final molarity noted and clotting times determined as in the preceding experiment

Results are given in Table III They show the usual (p 19) accel erating effect of certain small concentrations of calcium ions

Conclusion The usual acceleration of clotting in a certain range of calcium salt concentrations here 0 001 to 0 01 molar is evident There is a definite optimum at 005M with all three fibrinogens It must be concluded therefore that the Lorand serum factor is not significantly concerned with these effects of calcium ions on thrombin fibrinogen clotting times

2 EXPERIMENTAL EVIDENCE THAT CALCIUM IS NOT AN ESSENTIAL COMPONENT OF THE THROMBIN FIBRINOGEN REACTION

Since the careful work of Hammarsten [206] (see p 7) it has been reasonably established that calcium salts i e Ca^{++} ions are not essential to the thrombin clotting of fibrinogen Howell s comment [238] that there might still be room for doubt however indicated the need for final proof that the trace of (protein bound) calcium in the clotting reagents (fibrinogen as well as thrombin) is without specific influence on the clotting reaction

Experiment(s) 3 (1937 56) (cf Table XX)

Purpose To study the clotting of a completely decalcified thrombin fibrinogen system

Methods (A) in a 1937 experiment [128] the author prepared thrombin from Howell type prothrombin (p 90) by maximal activa tion ('ripe ' after several hours) with calcium and cephalin In three experiments this thrombin was electrodialyzed for 2 hrs 8 hrs 13 hrs respectively in a Bradfield 3 compartment electrodialyzer

by the author recently which deal with certain aspects of the serum (fibrin) factor problem (cf pp 20 21)

Experiment 1 (1956) Table II Figure 13

Purpose To investigate possible replationships of thrombin fibrinogen clotting times to the 'serum (fibrin) factor' of Laki & Lórand

Reagents and Methods

Preparation of 'serum factor' free fibrinogen (ref Laki & Lorand [277]; Lorand [304 305 307] Shulman [439] Stock fibrinogen: 1% Armour's bovine (plasma) Fraction I adsorbed with $BaSO_4$ to remove traces of prothrombin etc (p 55)

Fibrinogen I (Control): Stock fibrinogen dialyzed 2 hrs against 2M sodium chloride followed by overnight dialysis against 0 85% NaCl (room temperature) Thrombin clots of this solution remained insol uble in equal volume of 3M urea whether NaCl or $CaCl_2$ was added

Fibrinogen II Stock fibrinogen dialyzed 2 hrs against 2M sodium bromide followed by overnight dialysis against 0 85% NaCl The bromide treatment removed much of the serum factor but the test clots were still only partly soluble in urea in the presence of NaCl and negligibly soluble in the presence of $CaCl_2$

Fibrinogen III Fibrinogen II a week later was diluted 20x with distilled water and isoelectrically precipitated with 0 1M acetic acid with maximal turbidity at pH 5 5 The precipitate was recovered by 30 min centrifugation at 350 r p m and redissolved in 0 85% NaCl to original volume Thrombin clots were now nearly all soluble in 1 5M urea even with added calcium only a trace remaining after 24 hours

Testing fibrin clots for solubility in urea 0 2 ml Fibrinogen + 0 05 ml 0 1M $CaCl_2$ (or 0 85% NaCl) + 0 05 ml thrombin (bovine Up john s 10 units/ml) treated 10 min later with 0 3 ml 3M urea

Clotting time tests with the above three fibrinogens Clotting times are measured with a stopwatch at constant temperature (25°C) on adding to 0 2 ml of the respective fibrinogens 0 2 ml of bovine (Upjohn s p 56) thrombin in siliconed tubes which are used in order to avoid any loss of thrombin by adsorption onto glass The thrombin was actually added at twice the strength of the final concentration stated in Table II

Results are shown in Table II and in Figure 13 In the graphic pres entation in the cited figure relative thrombin concentrations are plotted against the reciprocal of the clotting times (1/c t)

Conclusion There appears to be a much better linearity in III and some improvement in II as compared with I (Figure 13) sug gesting that the 'serum factor of Lorand may indeed be a variable in causing deviations from the 'inverse law ' It is not known how valid the criterion of urea solubility of recalcified clots is in determining freedom from the Lorand factor It is possible therefore that further progress may be made along the present lines There is an unexplored possibility that disturbing factors other than the Lorand factor may

silicone Series I plasmas were kept in silicone but tested on both types of surface Series II were (G+) treated with powdered glass for 1 hr at 34°C in order to obtain a large measure of action of wettable surface The control series II (S) were held in silicone for the same period at the same (34°C) temperature Besides the Ca the following additives were tested: (1) saline (2) intact platelets (carefully pre served in silicone) (3) platelet extract (frozen thawed and triturated with saline) (4) brain cephalin In another series of experiments simply conducted in glass (pyrex tubes) (1) was repeated and tests were also made with (5) tissue thromboplastin (Soluplastin) (6) crys talline trypsin (100γ) (7) Loomis' [302] bovine fibrinolysin (4 mg) The data are shown in Tables IV and V

Results Table IV: In (1) with Ca and saline only no clots were obtained in 24 48 hrs whether in glass or in silicone This unequiv ocal result is difficult to achieve and was repeated on only two or three occasions In many more similar experiments clotting did occur in glass in from one to many hours and longer in silicone

In (2) and (3) clotting was restored with insignificant differences due to the manner of treating the platelets There was however a con sistent and significant difference between the tests on plasma only in contact with silicone and those exposed either (G) immediately or (G+) over a period to wettable glass surfaces The glass clotting times (170 210) may be compared with the silicone clotting times (435 660) The somewhat longer c t 's in the 1 hr experiments probably mean some loss of labile factor (AcG) That the plasma is actually altered by the surface contact is shown by the finding of the shorter clotting times also in the tests in silicone (II S) after trans fer to such tubes of the (G+) glass incubated samples

In (4) cephalin is shown to act in much the same way as platelets Actually it is even more effective in the concentration studied The accelerator effect of glass on some component of the platelet free plasma is also evident in the cephalin experiments

In the Table V data (1) the non clotting control is repeated (5) tissue thromboplastin is observed to give the very rapid clotting time of 7 sec exactly corresponding in fact to the typical prothrom bin time (Quick test) on ordinary dog plasma; (6) trypsin and (7) fibrin olysin are also effective That the two proteolytic enzymes of these last tests show similar 'thromboplastic' properties is an interesting observation which will require further investigation (see later)

Discussion None of the earlier efforts to establish the essential role of platelets in normal blood clotting were sufficiently convincing Fuchs [178] in 1930 claimed the obtaining of a spontaneously incoag ulable human plasma by high speed centrifugation in paraffined glass ware but Feissly (1934) and Smith Warner & Brinkhous (cited [64]) were unable *to confirm this work Introducing the silicone technique* in 1946 Jaques Fidlar Feldsted & MacDonald [251] noted the pro longation of natural and recalcification clotting times as platelet counts were more and more reduced by increasing centrifugation in silicon ized tubes However they were unable to get rid of all platelets and did not obtain an incoagulable plasma In the following year Brinkhous

with collodion membranes The 110 volt direct current reached a maximum amperage of 0 3 to 0 4 amps in ¼ hr and fell to 1 or 2 milli amps in about 2 hrs Distilled water was run continuously through both electrode (end-) chambers The thrombin began to floc culate out at the anode membrane of the middle chamber in about 15 min and to sediment Later it underwent partial migration to the cathode membrane The diphasic behavior was explained by a change in pH of the unbuffered material over to the acid side of the isoelectric point of the globulins present The final precipitate was recovered by centrifugation and redissolved in dilute Ca free KOH to pH 7 5 (phenol red indicator) The water clear supernatant always showed some relatively weak thrombic activity Neither the redissolved precipi tate nor the supernatant showed the least trace of turbidity on adding test reagent pot oxalate and spectroscopic tests for traces of Ca were negative The test fibrinogen was obtained from Berkefeld filtered (see p 69) citrated dog plasma 'deprothrombinized' by $Mg(OH)_2$ adsorption [176] and precipitated (x3) at ¼ sat $(NH_4)_2SO_4$ reprecipitating at ½ sat NaCl and redissolving in dist water We were unable to electrodialyze the fibrinogen without denaturation but relied upon excess of oxalate or citrate to decalcify it as indicated by a neg ative spectroscopic test for calcium The most crucial test was per formed after 18 hrs incubation of both (a) the 13 hr electrodialyzed thrombin and (b) the purified fibrinogen with equal volumes of (1) N/1 pot oxalate (2) N/1 sod citrate On mixture of 1 ml amounts of thrombin and fibrinogen thus treated and still in the presence of the strong inhibitory and decalcifying salt(s) firm clots were obtained in about ½ hr

An interesting incidental observation was clot lysis after several hours indicating activation of the fibrinolytic enzyme system contam inating the thrombin preparation

(B) Decalcification of proteins has subsequently become very simple with the advent of the ion exchange resins (p 275 of Stefanini & Dame shek [453]) We have repeatedly subjected both thrombin and fibrino gen to the cationic exchange resins Amberlite and Dowex '50' (sodium treated) and observed perfectly normal clotting of the subsequent mixtures (see Table XX)

Conclusion *It is therefore concluded that protein bound calcium* while contributing (like sodium potassium etc) to the charge patterns on the protein molecules is not essential for the thrombin fibrinogen reaction and that thrombin in no true sense is a calcium compound

3 EXPERIMENTS ON THE CLOTTING OF PLATELET FREE PLASMA

Experiment 4 (1949) [147] Tables IV V

Purpose To study the clotting properties of platelet free plasma

Method Citrated dog plasma was obtained with the siliconed tech nique after 16 hr centrifugation at 20 000 r p m in the refrigerated Multispeed ' Equal vols of each plasma sample were tested at 38°C for clotting times with 0 025M $CaCl_2$ (G) in glass and (S) in

preparing from it It must be reported that the advances to date while encouraging still leave much to be desired Nevertheless this should turn out to be a promising line of investigation leading toward the goal of purified individual blood clotting factors for specific therapeutic use in human deficiency cases

4 EXPERIMENTS WITH THE BERKEFELD (BACTERIOLOGICAL) FILTER TECHNIQUE (cf Experiment 2)

In 1913 Cramer & Pringle [95] claimed that the passage of citrated plasma through a Berkefeld filter rendered it incoagulable on subsequent recalcification but restored to clotting again on the addition of platelets They concluded that they had (a) discovered a simple technique for 'deplateletizing plasma and (b) proved that platelets were essential for normal clotting

W H Howell at once set one of his pupils C H Goddard (1914) [192] to repeating this experiment On carefully perusing the English data Goddard noted that some of their Berkefeld filtered plasmas did clot on simple recalcification although delayed perhaps for several hours One dog that had been fed three hours prior to bleeding gave a filtrate which clotted readily in 5 min i e practically a normal recalcification clotting time Goddard performed many Berkefeld filtrations and divided the filtrates into (1) first portion (2) intermediate and (3) last portion It was found that only the first portion which was slow in appearing through the fine pores of the diatomaceous filter showed marked clotting defects In fact it was often incoagulable even with thrombin proving that most of the clotting factors even the fibrinogen had been removed The last portions always clotted readily on simple recalcification The intermediate portions varied and not uncommonly would clot poorly if at all on adding calcium but quite well if platelets brain extracts or cephalin were also added Prothrombin could be recovered by Howell s acetone method even from some of the intermediate filtrates which did not clot satisfactorily with Ca alone

For a number of years the present author [126 130] routinely employed the Berkefeld filtration of plasma prior to preparing Howell type prothrombin By the simple bacteriological laboratory trick of inverting a large test tube over the candle the filtration was invariably rapid and satisfactory Test results confirmed Goddard in the finding of good clotting on simple recalcification Clotting times might be as long as 10 12 min instead of the usual (unfiltered) plasma s 2 3 min but could easily be restored to normal or better by addition of a small amount of cephalin [86]

Conclusions The previously cited more recent work with the silicone technique and high speed centrifugation undoubtedly proves that the present author s views of an earlier period were erroneous in believing that all platelet material could be removed by Berkefeld filtration We were looking for some plasma thromboplastin of other than platelet origin and well remember Howell s caution (p 480 of ref [233]) that platelets may very well disintegrate when roughly handled in ordinary centrifugation and in Berkefeld filtration There were important errors in Howell s other views however

[64] did succeed in obtaining a platelet free human citrated plasma with the silicone technique refrigeration and repeated centrifugations over a total period of 1350 min using speeds up to 14 000 r p m The reported final preparation recalcified in glass at 28°C did not clot in 30 hrs Prothrombin conversion was delayed in these 'quasi hemo philic' plasmas but prothrombin assays were normal 'Thrombin topical' and 'thromboplastic extract from beef lung' clotted them as rapidly as *normal whole blood Addition of platelet suspensions* reduced the clotting time to 6 9 min They destroyed added thrombin normally The platelet free normal plasma was able to correct the clotting defect of hemophilic whole blood but not of recalcified platelet free hemophilic plasma Correction did occur in the plasma mixtures however if some platelets whether normal or hemophilic were provided Hartmann & Conley [209] after many failures finally succeeded in obtaining a platelet free incoagulable canine plasma

Patton Ware & Seegers [369] used a special technique in collect ing blood without anticoagulant from a dog for siliconed tube centri fugation in the refrigerated 'Multispeed' at 22 000 23 000 r p m for 5 min - 5 hrs Some of the native' plasmas thus obtained remained fluid for longer than 72 hrs at room temperature There would usu ally however be some fibrin formed whether in glass or in silicone 'with about equal frequency Prothrombin analyses immediately after the centrifugations showed normal values compared with an oxalated plasma sample obtained at the same time as the special blood collection The more highly centrifuged samples retained the same assay levels of prothrombin for several hours Special (indirect) tests however showed the formation of traces of thrombin The fibrin noted represented only a small portion of the fibrinogen

Conclusions Our experiments [147] therefore confirm the few previous workers evidence pointing to the fact that platelets are nor mally essential for blood clotting The data on 'surface' effects caus ing some alteration of the platelet free plasma are original as is our use of cephalin and the enzymes in this type of experiment The fact that a lipoid (cephalin) and the other cited reagents can replace plate lets in what we believe to be a 'thromboplastic' type of action (see later) opens up some very intriguing questions

Addendum Owing to the technical difficulty in obtaining large quan tities of completely platelet free plasma the author (and others) have not progressed to the logical procedure namely to use such material as the starting point for the attempted preparation of plasma clotting factors free from all traces of natural 'thromboplastin' (at least of platelet origin) Mere 'platelet poor plasma is insufficient for satis fying this rigid criterion although the greater the success in this di rection the better *would seem to be claims for purity in preparing* prothrombin proconvertin proaccelerin antihemophilic globulin PTC and other factors As a member of the Coagulation Committee of the Harvard Plasma Fractionation Commission the author has followed the recent use of the ADL Cohn Fractionator in the preparation of deplateletized human plasma [460] and has tested some of the fractions (e g *prothrombin accelerator globulin* etc) which Dr Surgenor is

room temperature with an equal volume of benzene (C_6H_6) in a separatory funnel recovering the clear bottom layer No turbidity noted Eluate Sediment from the $BaSO_4$ adsorption (100 mg/ml) of the above plasma No 126 washed 2x with dist water and then eluted for 10 min with 1/3 orig vol of 0 2M sod citrate Aliquots of this eluate were (a) preserved frozen untreated (b) dialyzed 3 hrs against saline and (c) benzene extracted (b e) in a similar manner to the b e fibrinogen (see above) Another portion (d) was benzene extracted without dialysis and then (e) a fraction of this was dialyzed Specific assays of components of eluates (a) (d) were obtained by the routine methods previously described The data are given in Table VI and are expressed as a unitage/ml compared percentagewise with the statistical normal human plasma by which each test is standardized cf Figure 16 These eluates (1) lack fibrinogen and thrombin (no clot in 3 days in control tests) (2) contain very small amounts of AcG (proaccelerin) but are rich in (3) prothrombin and (4) proconvertin The dialysis and benzene extraction did not alter the prothrombin and proconvertin values significantly The high ionic strength of the 0 2M citrate is inhibitory in clotting tests unless the eluate is highly diluted It may be recalled that it is routinely diluted 50x in our eluate method III assays (p 59) Some incubations with 40 u/ml thrombin were performed with the undiluted eluates and failed to show any significant inactivation of the added thrombin There was however a 2 3x lengthening of clotting times from the start as compared with a 0 85" NaCl control With 0 2M sod citrate as the control however the clotting times (of the order of 25 30) were comparable A more significant antithrombin assay was therefore conducted on the b e dialyzed eluate (e) The data are given in Table VII with details of method appended There is no more than the merest trace if anything of progressive increase in clotting times in the test (2) with the eluate which is nothing like the rapid activation in defibrinated plasma or serum or in (3) the untreated AcG preparation I used as a positive control (in Table VII) and compared (4) with a partly purified AcG† described in Experiment 10 on p 76 AcG Our regular $BaCO_3$ treated bovine serum (p 58) assays about 1200 percentage units of (pro)accelerin/ml When assayed at full strength by the improved 2 stage method (p 59) it showed a barely assayable 1 unit/ml of thrombin yield It is probable that traces of both thrombin and prothrombin persist in barium carbonate treated bovine serum but they are quite negligible in the dilutions usually employed However we did attempt to purify this AcG preparation by an additional $BaSO_4$ adsorption This reduced its AcG unitage to 900 percentage units/ml but the traces of thrombin and prothrombin persisted as shown by the control tests (1 4) in the experiments of Tables X and XI Ceph Cephalin was prepared from acetone dried dog brain (cf Eagle [110]) by several days extraction with ether removing the acetone soluble fraction and resuspending the dried acetone insoluble lipoids in saline as a stock 3" suspension uniformly dispersed with aid of a mechanical homogenizer Dilutions (e g 0 5") from this stock are stable and uniform The stock 3" solution like other reagents is stored frozen at 20°C in the 'Deep Freeze This cephalin served as the standard in many experiments (e g p 98) Ca The optimal $CaCl_2$ concentration

5 EXPERIMENTS WITH THE BENZENE EXTRACTION TECHNIQUE AND ITS RELATION TO 'AVAILABLE' CEPHALIN

Apart from the errors in his work on platelets and lung extracts C A Mills [335 336] correctly controverted Howell's view that cal cium alone could activate prothrombin Mills insisted on an essential role for cephalin acting along with calcium as a 'thrombokinase' in the sense of the Morawitz [345 346] theory (see p 8) His evidence [336] included the following experiment:

Dog plasma was precipitated at ¼ sat $(NH_4)_2SO_4$ for fibrinogen followed by ½ sat with the ammon sulphate for a globulin fraction which served as a 'prothrombin ' after both fractions were dialyzed free from excess salt On extracting his prothrombin by shaking with benzene (C_6H_6) Mills obtained a preparation which when mixed with fibrinogen and calcium salt no longer gave a clot Coagulation was restored however on further addition of cephalin

Experiment 5 (1934) [123]

The present author's first modest essay into the blood coagulation field in 1934 was an attempt to confirm this experiment We were able to do so however only after benzene extracting (b e) the fibrin ogen also With considerable difficulty the recalcified mixture of b e fibrinogen and b e prothrombin could also be induced to clot by (a) restoring the benzene extractives (recovered by evaporation) in a watery suspension of the mere trace of material recovered or (b) layering the benzene extract on the top of the test mixture

Conclusion Of course an elementary knowledge of the lipid chem istry of the blood precludes any notion that this simple use of benzene can possibly extract more than a very small fraction of the fatty ma terials in the plasma fractions Indeed the paucity of material in our evaporated extracts bears witness to this Nevertheless the experi ment does show the removal of some plasma property which can be restored by added lipoid (cephalin) and which would appear to fit the definition of a 'thromboplastin' (see later) Re evaluation from the modern viewpoint raises at least one important possibility namely that the test materials are perhaps deprived of some labile clotting factor (? proaccelerin) not necessarily related to the partial lipoidal extraction This possibility will be explored in the present thesis

Experiment 6 (1956) Table VIII with assays on ELUATES (Tables VI VII)

Purpose To observe the clotting properties of an artificially iso lated system of identifiable coagulation components after subjection to benzene extraction

Reagents Fibrinogen (Fibr) Precipitated at 1/4 sat $(NH_4)_2SO_4$ from $BaSO_4$ adsorbed oxalated dog plasma redissolved in 1/3 orig vol of saline (0 85% NaCl) and dialyzed against saline containing 0 005M sod citrate Fibr. (b.e.): Fibrinogen shaken for 15 min at

Experiment 7 (1956) Table IX

Purpose To study further the clotting phenomena in 1 stage systems such as those of the preceding experiment and to test for clot promoting activity in the benzene extractives

Preparation of Extract (Extr) Preliminary observations gained from evaporation of the benzene layer from the preceding extractions indicated the extremely minute yields of extractives It was therefore attempted to obtain a more significant amount of material from whole plasma After 33 ml of a citrated dog plasma which had kept well for many months in the Deep Freeze' was thawed filtered (from trace of sediment) and extracted as usual with an equal volume of benzene Centrifugation of the top layer separated 30 ml of clear benzene from a small foamy deposit The clear extract was evaporated to dryness in vacuo and the residue extracted with 3 ml imidazole buffered saline Although this was definitely turbid it did not contain a weighable amount of extracted material

Method In a 1 stage test series similar to those of Experiment 6 the extract (extr) was compared with simple recalcification of eluate + fibrinogen mixtures An optimal amount of cephalin was substituted in another parallel test

As an independent observation the effects of b e AcG (a second extraction of the same reagent as used in Experiment 6 five days earlier) were studied both with and without cephalin

Results These are given in Table IX In (A) an eluate which had been benzene extracted and then dialyzed 5 days previously was used In (B) this same reagent was submitted to a further benzene extraction and tested twice namely (I) within 15 min and (II) after about 3 hrs Both series of tests employed the same freshly benzene extracted fibrinogen Note the following

(a) simple recalcification times show a significant prolongation after the fresh benzene extraction B 2 vs A 2 However there is some reversion to a shorter clotting time in the 3 hr test of B 2 after standing several hours at room temperature
(b) the extr reduces the clotting times although not as well as cephalin does in the AcG poor systems
(c) AcG gives considerable aid to the clotting system but only moderately so with Ca only and much more so when a relatively adequate thromboplastic action is restored by cephalin

All tests are consistently shorter in the A series The precious extract was not tested with AcG in order to conserve it for the more significant 2 stage experiments to follow

Conclusions Any conclusion from a 1 stage type of experiment must be tentative but it does seem reasonable to conclude that at least four factors are contributing toward the observed clotting times

was carefully determined for the eluate clotting tests by a preliminary series of mixtures corresponding to (8) in Table XI but with varied calcium concentration The cited amount of $CaCl_2$ was found to be optimal

Experiment Clotting test mixtures of fibrinogen (b e) and dia lyzed eluates (as described above) constituted in essence a synthetic plasma system of known composition and free from overt inhibitors Upon such the effects of benzene extraction could be studied in the clotting tests summarized in Table VIII

Results The most significant finding was the much poorer clotting time (1005+) in (5) with the b e eluate activated with (added) Ca alone than in the same eluate (9) before benzene extraction (354) Moreover the + sign indicates the timing of the first wisp of fibrin and many more minutes were required for a solid clot in (5) whereas (9) progressed to a solid gel' within a minute or two Recalcification in the presence of b e AcG ($BaSO_4$ treated) (7) considerably reduced the c t (210) showing that AcG lack was significant in the eluates as was known from the analyses in Table VI Test (8) showed cephalin to be a good (? partial) thromboplastin in the presence of Ca and AcG In comparison with (5) test (6) was equally significant in showing that cephalin could accelerate clotting even in a system very poor in AcG *In fact the 221 c t in (6) was better than the 354 c t in the simply* recalcified untreated eluate (9) All four tests with the untreated elu ate (9 12) were definitely better than the corresponding tests (5 8) with the b e eluate The differences between 11 (107 5) and 7 (210'') are especially significant because both are performed in the presence of adequate accelerator factor It should be noted that the 90 percentage units of AcG supplied in the 0 1 ml introduced into the total 1 ml mix ture corresponded very well to the level normally encountered in plasma Actually our synthetic system was hypoprothrombinemic (about 17 'percent') and hypoproconvertinemic (about 11 'percent') compared with a normal plasma The 16 7 c t in (13) and 15 7 in (14) where tissue thromboplastin (Soluplastin') was added with AcG and Ca and the temperature raise to 37°C are of the order of magni tude that would be expected if we were testing a plasma of similar composition by the regular 1 stage 'prothrombin time' test Tests1 4 are essentially controls for thrombin and prothrombin in the AcG preparation Thrombin is really absent from the eluate (see Table VI)

Conclusion These new experiments afford significant evidence of a diminished coagulability after benzene extraction It is essentially independent of the presence or absence of abundant accelerator factor (AcG) although this has its own effect on clotting time It is not an inhibitory phenomenon It can be corrected in fact over corrected by the addition of cephalin or tissue thromboplastin May it not therefore be concluded that (a) benzene extraction removes some thromboplas tic' factor associated with the complex proteins of the clotting system and (b) benzene being a lipoid solvent may not this factor be a throm boplastic lipid related to cephalin?

these systems particularly with Ca alone (2) The 'extract' adds a little which is significant and also indicates the extreme sensitivity of the test system (3) Although tests (1) and (2) are intentionally conducted with only the trace of contaminant AcG (see analyses in Table VII in the eluate the provision of adequate accelerator in the form of 1 8 dil b e AcG still adds only a nominal increase in the trace of thrombin formation The important conclusion is that thrombin formation is extremely poor in a system adequate with regard to pro thrombin proconvertin calcium and AcG but deficient in 'thrombo plastin '

An incidental finding in these tests confirms the antithrombin tests of Table VII Note the excellent stability over 3 hrs of the very weak thrombins in tests (1) and (2) with eluate mixtures only whereas the 2 and 3 hr tests in the presence of the serum AcG are definitely beginning to lose potency despite the 1 50 dilution of its serum anti thrombin There is some loss in all in 48 hrs especially in (3)

Experiment 9 is similar to the foregoing but relies upon the dilution and a little extra calcium to overcome the citrate The test data given in Table XI show

1) The AcG preparation has a minute amount of thrombin yielding potency even without any eluate (4)

2) Eluate with Ca yields very little thrombin even with the adequate provision of AcG (5) (7)

3) When the system is completed by addition of a thrombo plastin ' whether (a) cephalin or (b) crude tissue tpln thrombin formation is rapid and optimal

4) Cephalin is remarkably effective and compares well with tissue thromboplastin as to activity (shortest clotting time) attained but is definitely slower (7 min instead of 2 3 min) in reaching this optimum In fact the end point comparison in (6) and (8) indicates practically complete thrombin formation in the cephalin experiment

5) Extra proconvertin in (9) and (11) affords no benefits which agrees with the analytical findings (Table VI) showing that the eluate already contains it in adequate amounts

Conclusion The major conclusion from the foregoing experiments is that an extremely important clotting function must be assigned to factors playing a 'thromboplastic' role Significant among such is the phospholipid cephalin Modes of action need explanation however

6 EXPERIMENTS ON THROMBIN FORMATION
(6 A) REFERENCE STANDARDS

Experiment 10 (1956) Table XII Figure 14

Purpose To obtain reference assays for thrombin yields on a series of dilutions of 'standard' (canine) eluate containing prothrombin and proconvertin

Reagents Eluate (No 127) was prepared by 0 2M citrate elution of the usual $BaSO_4$ adsorbate from oxalated dog plasma as previously described (pp 55 71)

These factors are (1) eluate (i e prothrombin + proconvertin) (2) Ca (3) AcG (4) a thromboplastic type of factor which can be supplied rather well by cephalin and weakly by the benzene extract The fact that benzene extraction does not result in a system completely unable to clot with calcium alone suggests that a small amount of some thromboplastic agent normally accompanies the reagents studied That non clotting with Ca alone may be recorded in other systems of benzene extracted reagents (Experiment 5) may possibly be explained on two gounds viz (1) too great an ionic strength i e inhibitory to weak thrombin formation (2) antithrombic inhibitors in the system able to remove any small traces of thrombin formation before they can induce visible fibrin formation The present synthetic system permits the detection of very weak thrombin formation by eliminating these two unfavorable circumstances

In so doing however it still permits observation of a quantitative difference which can be interpreted as an ability of benzene extraction to remove a small portion of the potential 'thromboplastic' material in the clotting materials From the nature of the extraction and the similarity to a weak cephalin in restoration of the clotting deficit may it not be surmised that we are dealing with a thromboplastic lipoid? It is also suggested somewhat theoretically that the reappearance of greater clotting activity on simple recalcification at time after this has been lessened by benzene extraction may possible indicate some 'free' form of lipid becoming liberated from the natural lipo protein complexes and being 'available' in this form for (a) the clotting reactions on the one hand and for (b) easy removal (in trace amounts) by the lipoid solvent on the other

Experiments 8 and 9 (1956) Tables X XI

Purpose To study the conversion of prothrombin to thrombin in variously activated eluates by a two stage test system with special reference to thromboplastic additives

Reagents The same as those used in the preceding one stage experiments

Method The 2 stage procedure is essentially that of our standard 'Method III (see p 59) Note that the salt concentration (0 85% NaCl) and pH (7 3) are physiologically equivalent to plasma Moreover the 50 fold dilution (0 1 eluate in 5 ml thrombic mixture) reduces the citrate content to a point of non significance However the chosen amounts of $CaCl_2$ are strictly optimal according to careful preliminary tests on rapidly activating test systems

Experiment 8 is performed on dialyzed benzene extracted eluate: (1) with Ca alone (2) with Ca + extr (p 73) (3) with Ca + extr + AcG (b e)

The composition of the thrombic mixtures and the clotting test data after varying periods of incubation up to 3 48 hrs are given in Table X

Conclusions (1) There is extremely little thrombin formation in

(6 B) ANTIHEMOPHILIC GLOBULIN IN RELATION TO THROMBIN FORMATION BY CEPHALIN AND BY TISSUE THROMBOPLASTIN

Experiment 11 (1956) Table XIII

Purpose To study the effects of purified antihemophilic globulin (AHG) on thrombin formation from eluate prothrombin in relation to cephalin and to tissue thromboplastin

Method Method III two stage procedure as in the foregoing experiments Standard volumes of reagents are given on p 60

Reagents Eluate and standard fibrinogen as in Experiment 10 The eluate provides prothrombin proconvertin and PTC (? etc) AcG: same purified preparation as described on p 76 used in 1:5 dilution AHG: a purified preparation of antihemophilic globulin from bovine plasma for which we are indebted to our colleagues in the Pathology Department Dr R H Wagner et al [488] Our assays for its very potent antihemophilic activity and for a trace of PTC are described in Experiment 34 on p 107 (Table XXXVII) The other specific assays showed no thrombin prothrombin or proconvertin and test (1) of Table XIII proves that it lacks thromboplastic activity It does contain citrate and a trace of fibrinogen The last causes a very small amount of clot when used in the 1:100 dilution of a 1% stock solution during the incubation of the thrombic mixtures It also interferes with tests for antithrombin Ceph : 0 1% diluted from the same 3% stock cephalin solution (p 71) as in the preceding experiments Tpln : 'Soluplastin ' Schieffelin's (p 56) used (a) undiluted (1:1) or (b) at 1 5 dilution Ca: 0 15M $CaCl_2$

Results See data of Table XIII The control (1) without added thromboplastin but in the presence of AcG and AHG shows very little thrombin formation The trace activation is still continuing at 1 hr but is not significantly increased at 18 hrs (2) AHG with thromboplastin (1/1) shows little if any difference from the 'AHG free' control (3) i e the same maximal activation (15 9 c t) takes the same 3' time (4) AHG with thromboplastin (1/5) again shows no significant differences from the control (5) Both require 10 min for maximal activation and the slight difference in end-point clotting times is experimental and actually not in favor of the AHG containing mixture The trace of fibrin in the T M could be removing a little thrombin A very different result is seen in the cephalin experiments (6) with AHG and (7) without it There is a definite improvement in activation rate (7 min vs 15 min) and also a decidedly better (15 7 vs 25 5) thrombin yield when AHG is present

Discussion The circumstances in test (6) permit the interpretation that we are in basic principle performing by a new method the

Specific assays on Eluate No 127: (1) thrombin 0 (2) anti thrombin 0; (3) prothrombin 573 standard 2 stage (p 59) units per ml or 191 on basis of 'percentage' (of normal standard human plasma) (4) proconvertin 36 'percentage' units by 1-stage method (p 59) (5) AcG 1 stage (p 58): trace only

Fibrinogen (dog standard) was prepared from the $BaSO_4$ adsorbed plasma of the same dog by the usual technique (pp 55 70)

AcG† The routine AcG preparation (p 58) namely $BaCO_3$ adsorbed bovine serum was partially purified by precipitation at 1/3 sat $(NH_4)_2SO_4$ redissolving in original vol of saline and dialyzing 2 hrs against saline in cold room (4°C) A 1 5 dil of AcG† by pre liminary test proved optimal for tests of Experiment 10

Assays of AcG† (1) The data included in Table VII (see p 71) show the very considerable removal of antithrombin by this procedure (2) specific AcG assay (1 stage p 58) shows considerable loss viz 555 'percentage' units per ml compared with an untreated sample of the original AcG preparation (1240 'percentage' units) but neverthe-less a very serviceable preparation (3) no proconvertin; (4) merest traces of (pro)thrombin

Thromboplastin Schieffin's 'Soluplastin' (p 56)

Ca 0 15 M $CaCl_2$ optimal in amount used

Method Standard Method III ' except for use of 1:5 AcG† and vary ing amounts of eluate Hence: Saline and eluate (3 8 ml) Imid buff (0 5 ml) 1 5 AcG† (0 1 ml) Soluplastin (0 1 ml) 0 15 M $CaCl_2$ (0 5 ml)

Results are given in Table XII showing the actual test data and graphically in Figure 14 plotting 'end point' (i e minimal) clotting times against relative strength of eluate 100 'percent in our stand ard technique refers to 0 1 ml eluate per 5 ml (total) thrombic mix ture (T M) Since this represents a 50 fold dilution of the eluate it will correspond to 573/50 = 11 5 standard 2 stage prothrombin units per ml of thrombin forming mixture

Conclusions This assay series is very satisfactory for measur ing differences in the thrombin yields between 150% and 1% ('per-centage' units relative) of eluate As the amount of prothrombin is reduced the thrombin yield as measured by the minimal clotting time endpoint is reduced (longer c t 's) The required incubation period (3 ± 1 min) is not significantly different however until the prothrombin is below 20 percent (of standard) With very weak e g 1 percent eluate the lengthening of the incubation period is signifi-cant This might be due to the usual salt concentrations becoming unfavorable at very low protein (specifically prothrombin and procon-vertin) concentrations That relative excess of salts delays thrombin formation and lengthens clotting times is illustrated incidentally in Experiment 12 (p 79) Alternatively the lowered concentration of ac-cessory factors (Stuart proconvertin) may be a sufficient explanation (see SUPPLEMENT)

(6 B) ANTIHEMOPHILIC GLOBULIN IN RELATION TO THROMBIN FORMATION BY CEPHALIN AND BY TISSUE THROMBOPLASTIN

Experiment 11 (1956) Table XIII

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Results See data of Table XIII The control (1) without added thromboplastin but in the presence of AcG and AHG shows very little thrombin formation The trace activation is still continuing at 1 hr but is not significantly increased at 18 hrs (2) AHG with thromboplastin (1/1) shows little if any difference from the AHG free' control (3) i e the same maximal activation (15 9 c t) takes the same 3' time (4) AHG with thromboplastin (1/5) again shows no significant differences from the control (5) Both require 10 min for maximal activation and the slight difference in end point clotting times is experimental and actually not in favor of the AHG containing mixture The trace of fibrin in the T M could be removing a little thrombin A very different result is seen in the cephalin experiments (6) with AHG and (7) without it There is a definite improvement in activation rate (7 min vs 15 min) and also a decidedly better (15 7 vs 25 5) thrombin yield when AHG is present

Discussion The circumstances in test (6) permit the interpretation that we are in basic principle performing by a new method the

essential reactions involved in the 'thromboplastin generation test' (see p 47) Cephalin is an 'incomplete' thromboplastin and does not yield thrombin formation either as *fast* as or--in the present concen tration (10γ/ml mixture) as completely as tissue thromboplastin With the AHG (etc) however it does complete the thrombin forma tion to the same (15 7 sec c t) end point and in a time (5 7 min) between that of the 1:1 tpln (3 min) and that of the 1 5 tpln (10 min) It is presumed that PTC (and possibly other plasma thromboplastic factors see p 27) are available from the eluate See Experiment 34 (Table XXXVII)

Conclusion This experiment gives some very important informa tion about the thromboplastic effect of the phospholipid cephalin in that it indicates *how a weak partial thromboplastin may be converted* into a more powerful complete thromboplastin when AHG (antihemo philic globulin) participates in the reaction(s) There is evidence of some antihemophilic factor contaminant of our dialyzed eluate prepa rations (Table XXXVII Experiment 34) This could explain why ceph alin acts as well as it does in test (7) for instance It may very well be that this is the significant difference between our eluates and Seegers' more purified prothrombin (p 142) with respect to activa tion by Ca + cephalin

Addendum Role of PTC? *We do not at present have a suit* able PTC preparation to investigate its probable participation (ac cording to other current knowledge) in the generation of 'complete' thromboplastin We may suggest however that it should be possible to study this in much the same way with the basic test eluate prepared from a very PTC deficient plasma Some of our [298] human cases would be the most suitable source of such plasmas It is just possible also that such might be prepared from a dog after administration of dicumarol tromexan or similar drugs We have some unpublished data on PTC lowering (cf [350]) in several human cases undergoing *such therapy Low levels of PTC were found in all such cases tested* but it is questionable whether low enough levels could be thus obtained for the suggested experiment without too much loss of prothrombin and proconvertin Eluates from PTC deficients gave the answer (v SUP PLEMENT)

(6 B_1) THE THROMBOPLASTIN GENERATION TEST

We shall not include the experience we have ourselves had with the 'thromboplastin generation test' (p 46) We do not use it as a clinical routine because we believe our more specific AHG and PTC (and anti AHG and anti PTC) assays give more significant quantitative information The thromboplastin generation test may be considered to have considerable practical value in differential 'spot' diagnosis of the hemophiliac diseases The test has a value in these disorders com parable perhaps to what the Quick (prothrombin time) test has in the different group of prothrombin and related deficiencies There are a number of current workers both in England and in America who are *experimentally studying variants of the thromboplastin generation test* in order to learn more about the basic mechanisms and possible 'inter-

mediates' in these reactions (ref Bergsagel & Hougie [40] cited p 48) We are content therefore to leave to others this particular *line of inquiry and merely to add our own confirmatory data to what* is now widely known [354] and used namely that cephalin can sub stitute excellently for platelets (see p 47) in the performance of many variants of the thromboplastin generation test We shall however re view some of our own experiments of many years ago and some more recent confirmatory data on certain aspects of the problem of 'inter mediates' in thrombin formation with particular reference to the phosphatide cephalin and the role of calcium

(6 C) A CALCIUM CONTAINING 'INTERMEDIATE' IN THROMBIN FORMATION?

Experiment 12 (1956) Table XIV

Purpose To decalcify a thrombin forming mixture at various times from start of prothrombin activation and to observe the effects on thrombic activity Sequestrene NA_2 chosen as the decalcifying agent

Reagents Sequestrene Na_2 ('Versene' or EDTA) Alrose Chemi cal Co Providence R I has a molecular weight of 372 and solubil ity in water at 26°C of 11 1 g/100 ml 100 g EDTA 'sequesters' 10 5 g of Ca^{++} 3 72 g dissolved in dist water to 50 ml volume = 0 2 M (stock solution) EDTA: 0 1 M was used in Experiment 12 Eluate (No 127) see p 75 AcG† partially purified see p 76 Ceph : 0 1% cephalin see p 71 Ca: 0 1 M $CaCl_2$ Fibr : Std (dog) fibrinogen see p 70

Method A special problem was presented by the high ionic strengths of the total salts in the mixtures used in Experiment 12 A compromise was made which permitted the obtaining of clotting times which were reliably readable Although prolonged by the high salt content they yet allowed the necessary control of the calcium

Thrombic Mixture 1 6 ml saline 1 0 ml imidazole buffer 0 2 ml AcG† (1:5) 0 2 ml ceph (0 1%) 1 0 ml eluate 1 0 ml $CaCl_2$ (0 1M) 28°C

Series (A) 0 15 ml T M + 0 45 ml fibrinogen sequestrene (i e 8 vols fibr + 1 vol 0 1 M EDTA) This is properly controlled to be equivalent in all salts to the final test mixtures in series (B) Error'

Series (B) After 5 min of incubation of T M (above) 1 2 ml was added to 0 6 ml sequestrene (0 1 M) and 0 2 ml samples tested on 0 4 ml (untreated) fibrinogen immediately' (15 sec) and at stated times thereafter

Series (C) After 68 min of incubation of T M (see above) another 1 2 ml was treated with sequestrene (0 6 ml) and 0 2 ml samples repeatedly tested as in (B)

Results Under these particular experimental conditions maximal thrombic activity was attained in the T M after 15 min incubation ac cording to test series (A) in which the T M was incubated alone and the EDTA added (to control the salt mixture) only with the fibrinogen

In series (B) the initial 5 min test immediately after adding EDTA to the T M was only very slightly longer than the corresponding test of the (A) series Thereafter however as incubation of the T M with the sequestrene continued thrombic activity was not merely arrested but actually underwent a very significant reversal to progressively longer and longer clotting times In series (B) the initial test was if any thing slightly better than the minimal clotting time of series (A) but the small difference may have been due to use of a fresh tube of fibrin ogen On incubation with the sequestrene followed for an hour there was no trace of the 'reversal' phenomenon seen in series (B) but the T M retained its activity with excellent stability

Discussion This experiment is a successful confirmation of an observation which the author first made in 1937 [128] using Howell type prothrombin + Ca + cephalin as the T M and repeated in 1947 [156] with a T M composed of Seegers' purified prothrombin + Ca + 0 25% tissue thromboplastin (from brain Squibb's) These data are reproduced on pp 82 84 1 M oxalate or 1 M citrate were used as 'decalcifying' agents The former produced a heavy precipitate of CaC_2O_4 and the latter a translucency of clots which gave difficulties in the reading of end points The clot timing was carefully performed however The sequestrene experiments have good end points An ex tremely important conclusion was drawn from the first presentation (1937) of the experiment namely that the results can only be explained by postulating an intermediate Ca containing 'complex' (of prothrombin + thromboplastic factor and we should now doubtless include the prob ability of the accelerator and convertor co' factors also) This 'inter mediate' is demonstrable only during the earlier phases of prothrombin conversion After a time thrombin formation is complete and the 'ripe' thrombin can be decalcified without altering its clotting potency Some evidence of a true reversal back to inactive prothrombin in the pres ence of excess of the decalcifying agent was allegedly adduced in the 1937 experiments (see pp 82 83 but cf p 86)

Ignoring the citrate in the eluate (which isn't quite fair since it amounts to 0 04 molar in the 5 ml T M containing 1 ml of 0 2 M sod citrate used in the eluate preparation) the following computation indi cates the excess (equivalents) of EDTA over the added (1 ml of 0 1 M) calcium: In 3 vols of EDTA treated T M the 2 vols of T M supply 2/3 x 0 02 M (1/5 of 0 1 M) Ca while the 1 vol EDTA supplies 1/3 x 0 1 M sequestrene Na_2 The relative molarities do not tell the full story however Because of the ability of Ca to displace some of the Na in the chelating agent 100 g EDTA actually 'sequesters' 10 5 g Ca^{++} Therefore the above amount of EDTA namely 24 8 milligrams per ml is actually enough to sequester 2 5 mgm Ca The added Ca per ml of mixture however is only 0 53 mgm Hence there is nearly 5 times as much sequestrene Na_2 added as would be required to remove all the added calcium even if the 0 04 M citrate played no part in the removal of Ca ions Even a slight insufficiency of EDTA leaving a small part of the calcium still ionized results in failure to show the 'reversal' ef fect Instead slow continued activation occurs in Series (B) under these conditions

Conclusion These experiments support the hypothesis that an 'intermediate' containing loosely bound calcium is temporarily present during the activation of prothrombin to thrombin The final (ripe) thrombin can be decalcified without loss of potency

Experiment 13 (1956) Table XV

Purpose To repeat Experiment 12 but with the following modifica tions (a) use dialyzed eluate in order to provide more favorable ionic strength conditions and (b) substitute tissue thromboplastin for cephalin Further to demonstrate whether the sequestrene reversed' mixture can be re activated by subsequent restoral of calcium etc

Method Follows the same general principles as in the preceding experiment Temperature: 28°C

Thrombic Mixture (T M) 7 2 ml saline + 1 0 ml imid buff + 0 4 ml AcG† (1:5 dil) + 0 4 ml 'Soluplastin' (1:10 dil) + 0 8 ml dialyzed eluate (No 127) + 0 2 ml $CaCl_2$ (0 1 M) Dial. El.: The dialyzed eluate assayed 90 percentage prothrombin units/ml and pro convertin 216 percentage units

Series (A): 0 15 ml samples of T M tested on 0 20 ml fibrinogen + 0 05 ml 0 2 M sequestrene Na_2 at stated incubation intervals Error'

Series (B): After 1½ min incubation of T M an 8 ml aliquot was added to a waiting 4 ml of 0 2 M EDTA and a second test series started at the 2nd minute now using 0 2 ml samples of mixture (B) to 0 2 ml untreated fibrinogen

Series (C): After 1½ hr the remainder of mixture (B) was trans ferred to a Visking casing (cellophane) bag and dialyzed 1 hr against three changes of 0 85" NaCl The casing membrane was tied so as to just enclose the material to be dialyzed with the result that there was no volume change during the dialysis Subsequently a 3 ml aliquot was mixed with 1 ml of 'calcium activator mix (Ca mix 3 0 ml imid buff sal + 0 8 ml AcG† (1:5) + 0 8 ml 'Soluplastin (1 10) + 0 4 ml 0 1 M $CaCl_2$) and 0 25 ml samples tested against 0 2 ml of untreated fibrino gen after successive incubation intervals

Series (D): As a control for test series (C) 0 08 ml dialyzed elu ate was substituted for saline in a 1 ml mixture similar to the 'calcium activator mix in proportions of other ingredients and diluted with 3 ml of buff saline just before adding the eluate and beginning the test series viz 0 25 ml samplings tested on 0 2 ml fibrinogen as in (C)

Results are given in Table XV Notwithstanding the more rapid and complete activation (in about 5 min in (A) and in (D) series) by use of tissue thromboplastin the thrombin formation could be interrupted after 1½ min by adding 0 2 M sequestrene (1/3 total vol) and 'reversed in a manner qualitatively indistinguishable from that observed in Ex periment 12 The initial clotting time in the (B) series was 108 4 In an 8½ hr test (not included in Table XV) the c t was 323 The attempt (series (C)) to reactivate incubate (B) by (a) Ca addition (not illustrated) or (b) activator mix (Ca mix) was unsuccessful Within the few seconds of experimental error all tests of the (C) serie

reproduce the (B) c t value (at 90 *min*) with which the dialysis started Only further c t lengthening (cf (B) at 185 min) was ar rested Nothing like the control series (D) was shown in (C)

Discussion Success in this rather tricky experiment depends upon careful consideration of (1) ionic strengths (total salt content) * (2) amount of sequestrene (3) rapidity of activation (depending upon amounts of AcG thromboplastic agent calcium and eluate in the T M) and (4) choice of a period during activation at which the EDTA can start its action before completion of thrombin formation since ripe thrombin (Experiment 12) is insensitive to sequestrene inactivation *In error controls (A) of Expts 12 13 got 1/4 instead of 1/3 EDTA

Earlier Data The following data are selected from some 1937 experiments published by the author [128]

Experiment 14 (1937) Table XVI

Purpose To study the effects of excess oxalate at various stages of thrombin formation

Method A two stage technique essentially similar to the foregoing

Reagents Pro Howell type prothrombin from Berkefeld filtered citrated dog plasma (see pp 90 92) No attempt was made at the time to assay its prothrombin content and nothing was then known about proaccelerin and proconvertin Ceph : 0 1% brain cephalin [126] pre pared according to Howell [235] Ca 0 1 N (i e 0 05 M) $CaCl_2$ Oxal N/1 $K_2C_2O_4$ Fibr Precipitated from $Mg(OH)_2$ adsorbed cit rated dog plasma with 1/4 sat $(NH_4)_2SO_4$ reprecipitated and again a third time now with 1/2 sat NaCl This fibrinogen was prothrombin free according to test with Ca + Ceph which gave no trace of clot at 38°C in 24 hours [126 128] T.M. 40 ml Pro + 4 ml Ceph + 4 ml Ca Test 0 5 ml samples with 1 0 Fibr after various incubation periods 38°C

Series (I) After about 11 min activation 10 ml T M was diluted with an equal vol distilled water (control test) and incubated and tested parallel with (II)

Series (II) After 12 min activation another 10 ml aliquot of T M was mixed with an equal vol of N/1 $K_2C_2O_4$ and 1 ml samples tested at intervals thereafter with 1 ml fibrinogen

Series (III) After 4 hrs activation of T M a third 10 ml sample was treated with oxalate and tested in a similar manner

Results These are given in Table XVI

Experiment 15 (1937) Table XVII

Purpose To study the effects of excess citrate at various stages of thrombin formation

Method and Reagents As in Experiment 14 except for substi tution of N/1 sod citrate (for the oxalate)

Results are given in Table XVII

Discussion (Experiments 15 and 16) The obvious differences between (1) 'fresh' thrombin (10 15 min incubated T M) and (2) 'ripe' (several hours old T M) in the progressive inactivation of (1) but not of (2) with a 50 100 fold excess of oxalate or citrate were noted [128] The only effect on 'ripe' thrombin as in the initial (¼ min) test on 'fresh' thrombin is a prolongation of clotting time (compared with the control) due to the immediate effect of the high salt content

The cited publication (p 758 of Ferguson [126]) includes mention of the following: By dialyzing 'fresh' thrombin which had been pro gressively inactivated by citrate to a clotting time of about 1 hour we have been able to check the inactivation and by recalcifying to restore the clotting time to a value almost identical with a control consisting of untreated prothrombin with a like amount of calcium and cephalin (Cf p 86)

Electrodialyzed 'ripe thrombin (see p 65) was also unaffected by oxalate or citrate except for the immediate effect of the high salt concentration which was exaggerated by the low potency of these par ticular thrombin preparations

The thrombic mixtures (T M) untreated were remarkably stable in these early experiments (p 760 of Ferguson [126])

Conclusion (cited from [126]) It is clearly indicated that calcium forms an intermediary complex (prothrombin cephalin calcium compound) This complex soon passes over into a stable thrombin The intermediary can readily be deprived of its calcium with resulting inactivation whereas the final coagulant can be prepared calcium free without significant loss in potency The mode of action of the so called decalcifying anticoagulants is now elucidated In addition to their classical effect of preventing clotting by depression of the ionization of the calcium salts necessary for thrombin formation they can progressively remove calcium from the intermediary com plex Once the thrombin is ripe or fully elaborated however calcium is now no longer an essential component and oxalation or cit ration ceases to affect its potency except for the usual non specific immediate effect

Experiment 16 (1947) Figure 15

Purpose To confirm the 1937 experiments (14;15) cited [126] but using one of Dr W H Seeger's purified' prothrombin preparations and substituting a weak tissue thromboplastin for the phospholipid (cephalin) This 1947 experiment was presented graphically in three publications [156 141 147] and is reproduced in Figure 15

Method Essentially similar to the foregoing

Series I (T_1) 13 5 ml Seegers bovine prothrombin (0 5%) + 0 75 ml tissue thromboplastin (0 25% Squibb s rabbit brain com mercial preparation) + 0 75 ml 0 1 M $CaCl_2$ Clotting tests: 1 ml 1% bovine fibrinogen (Armour's) + 0 25 ml borate buffer, pH 7 7 (45 vol 2 5% H_3BO_3 45 vol 0 5% NaCl 10 vol $Na_2B_4O_7$ 10 H_2O) + 0 25 ml T_1

Series II: Same T M but tested (second phase oxalate control) by substituting 0 25 ml M/1 $K_2C_2O_4$ for the buffer

Series III (T$_2$) 3 ml T$_1$ 5 min old + 3 ml M/1 sod oxal Clotting tests: 1 ml B F + 0 5 ml T$_2$

Series IV, (T$_3$) 3 ml T$_1$ 30 min old + 3 ml M/1 sod oxal Clotting tests: 1 ml B F + 0 5 ml T$_3$

Experiment 17 (1956) Table XVIII

Purpose Further study of the progressive inactivation of thrombin forming mixtures using cationic exchange resin as the decalcifying agent Attempted reactivation

Method Essentially similar to the foregoing (see Experiment 13 p 81)

Reagents Same dialyzed eluate (No 127) 'purified' AcG† etc as in Experiment 13 0 1% Ceph (p 71) B F 1% Armour's bovine fibrinogen adsorbed with $BaSO_4$ (p 55) Dowex '50'--sulfonic acid resin (Dow Chemical Co Midland Mich) charged in sodium cycle (p 275 of Stefanini & Dameshek [453]) T M : 13 ml saline + 2 ml imid buff + 1 ml AcG† (1:5) + 1 ml 0 1% ceph + 1 ml dial eluate + 2 ml 0 02 M $CaCl_2$ All clotting tests use 0 2 ml incubate + 0 2 ml B F at 28°C or (D) equivalent specified

Series (A): Control

Series (B): After 10 min incubation 10 ml T M is added to 1 g Dowex '50' and shaken vigorously at intervals thereafter with testing of clotting potency of the clear supernatant

Series (C): After 1 hr incubation another 5 ml of T M incubate is similarly treated with 0 5 g Dowex '50' and retested

Series (D): After 20 min shaking with Dowex '50' 2 ml of (B) is treated with 0 25 ml AcG† 0 25 Soluplastin (p 56) and 0 5 ml 0 02 M $CaCl_2$ Thereafter tests are made with 0 3 ml of (D) + 0 2 ml B F

Series (E) Original eluate pre treated with Dowex '50' (100 mg/ml) for 1 hr (next day) and used in T M of same composition as (A)

Results Given in Table XVIII show that the decalcifying resin gives an excellent 'progressive' inactivation in (B) when the thrombin formation is not quite complete as seen from the control series (A) The similar tests after 1 hr (series C) show much less effect of the decalcification That a little progressive inactivation does occur in this series may be regarded as evidence that the thrombin formation is not yet quite complete This is probably due to the use of the relatively slow acting and 'incomplete' thromboplastic factor provided by the 0 1% cephalin with eluate poor in AHF

It was not possible to demonstrate any re activation by restoring Ca and other 'activators' in series (D) although these clotting times were somewhat shorter than in the (B) series at the start of the (D) experiment There is possibly a point of some minor significance in the data (e g first test in (B) and (C) suggesting a slight 'immediate' inhibitory action of the Dowex '50' which might be due to some alteration of the ionic strength pattern in these mixtures)

Series (E) performed next day clearly shows that Dowex '50 has no effect on the original eluate After 1 hour s treatment with the resin the eluate activates just as well (if not better ? experimental) than in the control series (A) particularly reaching the end point in an identical (15 min) incubation period In other experiments (see Table XX) bovine thrombin and dog fibrinogen were subjected to prolonged treatment with Dowex '50 ' without significant changes in their reactivity

(6 D) A LIPID CONTAINING INTERMEDIATE IN THROMBIN FORMATION?

Experiment 18 (1956) Tables XIX XX

Purpose To study the effect of benzene extraction during the conversion of prothrombin to thrombin in eluates (prothrombin + proconvertin) activated by AcG cephalin and Ca salt

Method Essentially similar to the preceding experiment and using the same preparations of dialyzed eluate purified AcG† (1 5) cephalin (0 1") and buffered saline Ca (0 02 M) Armour's bovine fibrinogen (B F) 1" $BaSO_4$ adsorbed was used as substrate 0 2 ml fibr + 0 2 ml (or equivalent) of test mixture after successive incubation periods

Series (1): Control activation of the usual T M viz 6 5 ml sal + 1 ml imid buff + 0 5 AcG† + 0 5 ceph + 0 5 eluate + 1 0 $CaCl_2$

Series (2) After 5 minutes activation of (1) an aliquot was treated with an equal volume of benzene in a separatory funnel with vigorous shaking at intervals and 0 2 ml samples retested after further incubation

Series (3) After 70 min inactivation of (2) a 1 ml sample was re treated with an activator mix of 0 2 AcG† + 0 2 Ceph + 0 1 Ca and 0 3 ml samples tested thereafter

Results are shown in Table XIX The data clearly show a progressive inactivation in the b e mixture of incompletely formed thrombin (B) It was not possible to reactivate the 70 min sample of (2) either by the complete activator mix (C) or in other attempts (not illustrated) using (a) ceph alone or (b) ceph + AcG†

Discussion This new experiment is a most interesting piece of evidence to support the 1936 1938 suggestion [126 129] that free or available cephalin also participates in the postulated intermediary during thrombin formation The earlier benzene extraction experiments (pp 70 et seq) showed that fibrinogen eluate (prothrombin and proconvertin) and the AcG preparation could be shaken with benzene without more than minor changes in potency Control tests on a bovine thrombin (i e fully formed thrombin) shown in Table XX likewise demonstrate the essential insensitivity of thrombin to the benzene extraction technique With these controls therefore the progressive inactivation of partially formed thrombin (series (2)) is particularly significant

Conclusion The foregoing evidence then definitely points to a loose binding of 'free' phospholipid (cephalin) into some 'intermediary complex' during the conversion of prothrombin to thrombin Removal of such lipid by benzene extraction does not seem to restore the orig inal prothrombin (activatable by Ca ceph AcG) but to result in some inactive by product (? 'autoprothrombin ' in Seegers' sense)

SUMMARY OF DATA ON THE POSTULATED 'INTERMEDIARY' COMPLEX (Experiments 12 18)

That there is a progressive inactivation not merely an arrest of further thrombin formation when a partially activated thrombin form ing mixture is treated with a decalcifying agent has been repeatedly confirmed by experiments which we have made in 1937 1947 and again in 1956 In all we have used (a) three different types of pro thrombin preparation (Howell type Seegers type and our own 'eluate') (b) four types of decalcifying agent (oxalate citrate sequestrene and ion exchange resin) and (c) both partial (cephalin) and complete (tissue extract) types of thromboplastin On the other hand the completely formed or ripe' thrombin is essentially devoid of calcium and is not specifically affected by the decalcifying anticoagulants The progres sively inactivated mixture cannot be restored to prothrombin (activat able by the usual means i e Ca thromboplastin proconvertin AcG) Earlier (p 83) suggestion [128] that such reactivation might be pos sible must be reinterpreted as probably due (as was conceded at that time) to a considerable amount of the original prothrombin persisting unchanged through the test We were always able to increase the po tency of our electrodialyzed thrombin by further recalcification for instance The tentative suggestion is that the decalcified intermediary changes to an inert by product perhaps similar to Seegers' 'autopro thrombin(s) '

The new (1956) experiments demonstrate an apparently similar progressive inactivation when a partially activated mixture (prothrom bin + proconvertin + AcG + free cephalin + Ca) is treated with benzene whereas the final ('ripe') thrombin is unaffected by this lipoid solvent

These data support the hypothesis advanced by the author in 1937 namely that the conversion of prothrombin to thrombin involves an intermediary step during which the calcium and mobile phospholipid can be removed resulting in a progressive inactivation phenomenon The new addition to this theory is the suggestion that the removal of either Ca or cephalin causes the intermediary to alter its normal re action pattern (toward thrombin formation) and be diverted into some side reaction yielding an inactive by product (? autoprothrombin(s)')

(6 E) FATE OF PROCONVERTIN DURING THROMBIN FORMATION

Preliminary experiment In a repetition of Experiment 17 100 mg/ml Dowex '50' was added 5 min after the start of activation of the usual (A) T M (same mixture as on p 84) Just after the addition of the resin (B) the test clotting time was 20 9 After 1 hr with the Dowex '50' it has lengthened to 106 4 Standard one stage procon vertin assays (p 59) gave the following 'percentage values: (A) i e

untreated T M at start 110"; (B) i e 1 hr after adding Dowex '50 ' 54". This could mean merely that about half the original proconvertin was consumed (utilized) in forming the 5 min (? intermediate) thrombin and was surviving in the subsequent resin incubate The answer is certainly negative to the purpose of the inquiry namely to see if there was any increase in proconvertin activity as with some of Seegers' group's [13] experiments with 'autoprothrombin '

It remains for future investigation to explore further the possible connections between our line of approach and the quite different methods used in Dr W H Seegers' laboratories

(6 F) QUANTITATIVE RELATIONSHIPS OF CEPHALIN (VARYING CONCENTRATIONS) TO RATE AND YIELD OF THROMBIN FORMATION

When the prothrombin and proconvertin (in the eluate) the AcG c cium and saline buffer are kept constant our two stage Method III becomes a valuable quantitative technique for assay of thromboplastin according to the effects which their additions have upon the rate and yield of thrombin as determined by the clotting times (for a standard fibrinogen) in the second stage of the test Since these assays are relative it is necessary on each occasion to prepare a reference series of dilutions of some 'standard' at the same time and with all the same reagents as in the tests with the unknowns We choose as standards: (1) dog brain cephalin suspension (p 71) when testing various lipid preparations (2) 'Soluplastin' (p 56) when comparing various tissue thromboplastin preparations and (3) freshly obtained platelet suspension from a normal human donor (p 54) when assaying a patient's platelets for 'preformed' thromboplastic component (factor 1 in our listing p 50)

Experiment 19 (1955) Table XXI

Purpose To prepare a standard 'reference series of cephalin dilutions and to study the effects on rate and yield of thrombin formation by 'Method III as previously described

Reagents The prothrombin and proconvertin eluate is described on p 71 the untreated preparation being used in routine assays The AcG is described on p 58 and is further 'purified (p 76) only for specially sensitive assays where it is desirable to minimize any instability of the end point The usual saline imidazole buffer (pH 7 3) and $CaCl_2$ are used

The standard T.M. (thrombic mixture) 3 7 ml saline + 0 5 ml imidazole buffer + 0 1 ml AcG (1 8) + 0 1 ml thromboplastic preparation (to be tested) + 0 1 ml eluate + 0 5 ml 0 15 M $CaCl_2$ At successive incubation periods 0 2 ml T M is tested for the clotting times (at 28°C) with 0 2 ml fibrinogen (dog or bovine p 55)

Results are shown for an illustrative experiment in Table XXI

Discussion Some of the test series were not carried to completion

They are in fact a typical reference series taken at random from one of our routine studies in connection with investigation of the thrombo plastic potency of various lipoids (see p 98) They include one or two small imperfections to illustrate the occasional experimental error e g test series 4 is 'out of line ' By and large however they are re liable and serve to demonstrate the following:

(1) the smaller the concentration of cephalin the slower the rate of thrombin formation This is denoted by the sequence of clotting times and particularly by the incubation period required to reach the minimal clotting time end point

(2) the less the cephalin also the less complete is the thrombin yield This last is measured by the end point (minimal) clotting times in a relative manner and can be quantitated by use of refer ence standards of eluate dilutions such as those given in Figure 11 and Table XII

(3) while our cephalin is usually to be regarded as an incomplete thromboplastin especially when comparing activation rates with those in similar thrombic mixtures but substituting tissue thromboplastin (e g Experiment 9 p 75) the final thrombin yield in our test systems is often surprisingly good viz 90 100 percent in some cases and sel dom under 50 60 percent if optimal strength cephalin is used For suggested explanation see p 78

(4) that there is an apparent 'optimum' cephalin concentration is illustrated in Table XXI by the poorer yield (28 1 c t) despite the shorter (15 min) incubation period in the case of the 100γ strength (test 1) as compared with the 50γ (test 2) viz 22 ' minimal c t in 20 min incubation

That this is really due to the fact that cephalin is still a relatively crude preparation (see later) and contains some lipid inhibitor is now well recognized [73] Similar inhibition has also been reported for excess or certain fractions of tissue thromboplastin [4 91 358 359 193] and even for platelet suspensions [261] This immediately suggests however that our test can also be used to assay such antithrombo plastic inhibitors This we do simply by mixing the unknown material with an optimal (usually 50γ) cephalin and noting the reduced thrombo plastic potency

(5) The least cephalin concentration tested in this particular ex periment was 0 78γ or 1:1 280 000 (test 8) This is still very much superior to the control (0) with no added thromboplastin It is obvi ous therefore that the method is extremely sensitive and is well able to detect thromboplastic activity in lipid suspensions (such as cephalin) diluted to something of the order of one in several millions The self imposed limits of the illustrated test series easily suffice for screen ing tests on unknown lipids such as those to be described in a subse quent section (p 98)

Conclusion These assays are an exceedingly sensitive measure of the relative thromboplastic potency of (cephalin like) lipids Alter natively used with cephalin they are equally sensitive indicators of antithromboplastic (anticephalin) inhibitors The cephalin concentra tion determines both the rate of prothrombin activation and (with sub optimal strengths of cephalin) the final thrombin yield

Earlier Data While the Howell type prothrombin used in our earlier research was not as good and as well controlled as in the present recent studies it did suffice to establish the same fundamental conclusions as shown in the following data reproduced from a 1938 publication [129]

Experiment 20 (1938) Table XXII

Purpose To study the effects of varying cephalin concentrations on the rate and yield of thrombin formation

Reagents Fibrinogen Howell type prothrombin (Pro) Ceph and Ca(N/10) as described on p 82

Method Essentially the same type of two stage i e (1) activation and (2) testing with fibrinogen as in preceding experiments
T.M.: 10 ml Pro + 1 ml Ceph (cited final dilutions) + 1 ml Ca
Clotting Test: 1 ml fibr + 0 5 ml T M at successive incubation periods

Results are reproduced in Table XXII (Table 1 of cited ref [129] in which the data are also presented graphically in its Fig 2)

Discussion The results are essentially the same as those in Experiment 19 Instability of the end point was often troublesome with Howell prothrombins (see later) and there was the slight possibility that the differences persisting after 3 hrs incubation might be partly due to instability complications However this possibility could not explain the 4 hr tests where after a 5 min incubation with an additional 1/5 vol of 1 1000 cephalin all series attained a minimum c t of 12 The extra dilution of the T M in all probability sufficiently accounts for the 12 value It may be recalled that it was not until November 1939 that Mertz Seegers and H P Smith [332] published the results of their experiments showing similar relationships of tissue thromboplastin The two stage assays of prothrombin and thrombin gave much weight to the findings of these reliable investigators However we continued to believe that relative clotting times in carefully controlled comparative experiments were equally valid evidence of the phenomena under consideration

(6 G) EXPERIMENTS WITH TISSUE THROMBOPLASTIN

In 1948 we again performed the foregoing type of experiment but with highly purified prothrombin and using a tissue thromboplastin The following data are reproduced from that work [156]

Experiment 21 (1948) Table XXIII

Purpose To study the effects of varying concentrations of tissue thromboplastin upon the rate and yield of thrombin formation from highly purified prothrombin

Reagents Prothrombin (Pro A) was prepared by ourselves from citrated dog plasma following closely the procedures (for bovine pro thrombin) of Seegers Loomis & Vandenbelt [431] It corresponded to 'Product 4' (stage of purification) of the cited authorities and com pared very favorably with a number of Seegers' own (bovine) prepara tions with which we also experimented at that time [156]

Tpln. A: Squibb's (commercial) rabbit brain thromboplastin
Fibr 1% B F (Armour's bovine see p 55)

Method 5 ml vol T M containing 2 ml Pro A + 0 25 ml 0 1M $CaCl_2$ + tpln A (final concentrations stated in Table XXIII) in borate buffer pH 7 7 (see p 83) Clotting tests: 0 5 ml B F + 0 25 ml T M at 25 ± 2°C

Results are summarized in Table XXIII stating (a) the final tpln concentration (b) the end point clotting times (sec) (c) the incuba tion period needed to reach the end point

Discussion Seegers type prothrombins at that time undoubtedly contained proconvertin but probably only questionable traces of proac celerin (AcG) [494] However these factors were not generally known at the time these experiments were performed It was merely a de monstrable fact that in our experiments Seegers type prothrombins could be activated very slowly under unfavorable activation conditions e g with Ca alone or sometimes without any addition [146] Cephalin was a poor activator of Seegers' prothrombin [146] Nevertheless the stability of our end points often for 3 4 weeks at room tempera ture in the bacteriostatic borate buffer solution was indeed remark able The fact that small differences in the end point clotting times were apparent in the data (see Table XXIII) therefore did seem to confirm the fact that below a certain optimum ' lessening the thromboplastin definitely reduces the final amount of thrombin formed in addition to greatly slowing the rate of activation '

ADDITIONAL EXPERIMENTS WITH THROMBOPLASTIN tested on eluates (1956) qualitatively resemble the cephalin data (Experiment 19 Table XXI) and will not be cited in detail

Conclusion (Experiments 19 21) The author's data (1938 1948 1956) repeatedly confirm the apparent fact that the amount of thrombo plastic factor (whether cephalin or tissue thromboplastin) below a certain optimum (which may be a matter of contaminant inhibitor) de termines both the rate of prothrombin conversion and the final throm bin yield These ideas can be added to the 'intermediary theory very nicely but it does not seem appropriate to assume that the relation ships are 'stoichiometric ' Even if this last could be demonstrated for an 'intermediate it need not be true for the final thrombin

(6 H) SOME MODERN ASSAYS AND TESTS ON HOWELL TYPE 'PROTHROMBIN WITH REFERENCE TO FACTORS WHICH MAY AFFECT THROMBIN FORMATION

Because of some uncertainties in retrospect concerning clotting factors other than prothrombin which may have been present or

deficient in our earlier preparations of Howell type 'prothrombin' (with which many of the fundamental observations presented in this thesis were originally made) it seemed desirable to subject this re agent to the scrutiny of our modern assay methods

Preparation As in the past (1937 1938) citrated dog plasma (p 54) was subjected to Berkefeld filtration (p 69) and subsequently defibrinated by warming cautiously in test tubes held in a water bath at 55oC for two minutes then filtering through coarse filter paper Prothrombin papers were prepared from 5 ml quantities of the filtrate by the acetone method of Howell [235] and his pupil Cekada [71] This consisted simply of rapid precipitation with an equal volume of ace tone and immediate collection of the precipitate on a filter paper in a Buchner funnel under suction pump and quickly washing with a test tube full (about 20 ml) of ether and finally drying in an air current vigorously applied with the aid of a jet or electric fan

Prothrombin Solution (Pro) is obtained by cutting up the dry papers and extracting them with a suitable volume of di stilled water (usually 10 ml per paper) containing a drop or two of 0 5% $NaHCO_3$ and subsequently filtering The pH should be about 7 5 as tested with (a) phenol red indicator (b) pHydrion paper (Micro Essential Labs Brooklyn N Y) or (c) the (Coleman) glass electrode pH meter Borate buffer pH 7 7 (p 83) or imidazole buffered saline pH 7 3 (p 56) are superior extracting agents for the prothrombin papers The former was used in the following experiment

Experiment 22 (1956) Tables XXIV A XXIV B

Purpose To assay the principal clotting factors in Howell type prothrombin

Methods (1) Fibrinogen test; 0 2 ml Pro + 0 2 ml thrombin (bo vine Upjohn s 20 units/ml p 56) (2) Thrombin test 0 2 ml Pro + 0 2 ml fibrinogen (p 55) (3) Prothrombin assay by improved two stage method (p 59) (5) Proaccelerin assay by specific one stage method (p 58) (6) Antithrombin test: incubate with thrombin as de scribed on p 71 (Table VII) (7) (8) PTC and AHF assays p 107 Experiment 34 (cf Table XXXVII)

Reference Curves for the one stage assays were obtained on normal dog oxalated plasma dilutions and are shown in Figure 16

Results are shown in Tables XXIV A and XXIV B comparing the prothrombin (Pro) solution with the precursor plasmas

Fibrinogen: is absent from Pro (4) and (almost) from the heat defibrinated plasma (3) but of course is abundant in the original plasma (1) c t = 9 3 sec and in the Berkefeld filtrate (2) c t - 10 sec

Thrombin: traces are not infrequently (but not invariably) demon strable in Pro, (4) In the cited test a small trace of clot appeared in 3/4 hr but was still very incomplete after many hours

Prothrombin: The normal (100% : about 254 two stage units/ml) was found in (1) Nearly half was lost in the Berkefeld filtration (2) and in the heat defibrinated plasma (3) Only 10 15% levels were

found in the final Pro solution (4) Allowing for the double (cf plasma) vol of extract (10 ml per paper) the 'recovery' is about 20 29% of the original plasma prothrombin

Proconvertin: The original plasma (1) assayed a normal 96% About 1/3 was lost in the Berkefeld filtration (2) and about 1/2 after the heat defibrination (3) A 20% level was found in the Pro solution (4) which correcting for the dilution represents a 40% 'recovery' of the original plasma proconvertin

Proaccelerin The original plasma (1) assayed a normal 80% This was reduced to 25% after the Berkefeld filtration (2) and negligibly more (22%) after the heat defibrination (3) The amount (25%) in the Pro solution (4) corrected for the dilution represents a remarkably good 50% 'recovery' of the original plasma proaccelerin

Antithrombin: The test in Table XXIV B showed this to be essentially absent

PTC: According to the assays reported in Experiment 34 (pp 107 108) (dog) Howell prothrombin (1:1) is better than a normal (human) plasma (1 5) in correcting a known PTC deficiency These data suggest that the preparation contains most of the original plasma PTC

AHF: was also assayed in Experiment 34 (pp 107-108) It too is positive but in Pro (1 1) was less active than in 1:10 normal human plasma The data on these assays must be considered very significant

Conclusion Howell-type prothrombin preparations are not only rich in prothrombin but also contain significant amounts of proconvertin proaccelerin PTC and AHF They lack antithrombin and fibrinogen but may contain traces of thrombin These findings may very well explain why the old experiments with Howell-type prothrombin gave significantly correct answers to a number of basic questions concerning the conversion of prothrombin to thrombin

(6 I) *QUANTITATIVE RELATIONSHIPS OF CALCIUM IN THROMBIN FORMATION*

Experiment 23 (1956) Table XXV

Purpose To study the effects of decreasing calcium concentrations on the conversion of prothrombin to thrombin in dialyzed eluate (prothrombin + proconvertin) in the presence of adequate AcG and tissue thromboplastin

Method Two-stage technique similar to that used in previous experiments

T.M.: 0 2 ml eluate (No 127 dialyzed) + 0 1 ml AcG† (p 76) + 0 5 ml 'Soluplastin' + $CaCl_2$ (volumes of 0 02 M solution cited) + imid buff saline to total 10 ml vol The stock (aqueous) $CaCl_2$ solution was 0 1 M and all dilutions were made with buff saline

Clotting tests: 0 4 ml fibrinogen (dog) + 0 2 ml 'adjusted' Ca (to give a final conc of 5m M in the T M + F test mixture) + 0 2 ml T M, tested after successive incubation periods 26°C

Results These are given in Table XXV

Discussion The Ca conc was varied between 8 and 0 5 milli-Molar

in the T M keeping the other components constant The clotting tests were all conducted at a fixed (final) Ca conc of 5mM The effects of reducing the Ca concentration during thrombin formation were (1) a definite slowing of the earlier (e g 1 min) phases of activation but (2) no significant effect upon the final thrombin yield all c t end points were within a few tenths of a second of each other (4. 5 ± 0 3 sec)

The negligible activation of the control (6) may just be the trace of (pro)thrombin in the AcG preparation as noted on p 76

Experiment 24 (1956) Table XXVI

Purpose To study the effect of increasing calcium concentrations on thrombin formation and to investigate whether there is a Ca optimum for this reaction

Foreword In the author's [129] 1938 experiments on this subject using Howell type prothrombin and cephalin as the thromboplastic agent the effects of the variable amount of calcium carried over into the final thrombin fibrinogen reaction (cf Experiment 2 p 65) were not controlled This led to some just criticism especially by Wohlisch [506] who cited Weitnauer's [499] controlled experiments In Experiments 23 24 25 we have avoided this error by adjusting the final Ca conc in the clotting test mixture to a fixed value This is conveniently done by including a carefully computed additional buffered Ca salt solution with the fibrinogen just before the final clotting test With high concentrations of calcium in the thrombic mixture (T M) this results unavoidably in some excess of Ca in all the final tests However this is a relatively minor point compared with the control achieved in validating the results of the main experiment

Method Essentially the same and with the same reagents as in Experiment 23 except for the increased amounts of $CaCl_2$ 1 Molar stock (14 7% $CaCl_2$ $2H_2O$) was dissolved in imidazole buffered saline

Results These are given in Table XXVI

Discussion These experiments were very carefully performed with excellent reagents and apparently homogeneous and only slightly opalescent mixtures Clotting times were accurately determined with a stopwatch and the end points were sharp The results however show some unexplained variability which is considerably more than in experiments at lower (calcium) salt concentrations We like to think our experimental error in clot timings is well within 10 percent and indeed it is usually within a few tenths of a second in literally thousands of tests in a variety of two stage systems We are not quite sure therefore whether the variability of the end points (minimal c t s) in Experiment 24 has any real significance Accepting a ±10% experimental error the end points of I IV were all within 36±4 sec and of V (at 45 4) just a little longer than these limits There was certainly no very striking and consistent reduction in the thrombin yield on increasing the Ca in the T M from 8 to 25 mM The 8 mM Ca level was best in this series (increasing Ca conc) as in the decreasing Ca series of Table XXV There is therefore some evidence of a

calcium optimum at this 8 mM level Above this level the most con sistent effects of increasing the Ca are the longer c t 's in the one minute test just as is the case (Experiment 23) in reducing the Ca conc below this optimum level In the 5 min test however there appears to be some slight improvement with increasing Ca

Conclusion It would seem that the main effect of varying the cal cium level in our thrombin forming mixtures is to modify the initial rate of thrombin formation Effects on the final thrombin yield are very questionable at Ca levels above the optimum and negligible (if any) at sub optimal Ca concentrations

Experiment 25 (1948) Table XXVII

Purpose To study the effects of varying amounts of Ca^{++} during prothrombin activation using Seegers [431] 'purified' prothrombin in presence of tissue thromboplastin

Foreword This experiment of the author's was published in 1948 [156] In retrospect we were dealing with a very stable prothrombin undoubtedly containing proconvertin but very poor in AcG (hence the slow activation) The proper second phase controls were included

Method and Results are given in Table XXVII They demon strate 1) no activation (in 2 hrs) in the absence of added Ca 2) slower rate of activation if the Ca conc in the thrombic mixture is too low (2) or too high (4;5) but 3) no effect on the final (2 hr) throm bin yield i e identical (4 sec) minimal c t end points

Conclusions (Experiments 23 25) The cited experimental data permit the important conclusions that the rate of thrombin formation from prothrombin in the presence of adequate thromboplastin and co factors (proconvertin and AcG) is determined by the calcium con centration in the activating (T M) mixture Too little or too much calcium retards the thrombin formation and there is a definite Ca optimum at about 0 008 M in protein poor mixtures at pH 7 3 and 0 15 ionic strength However there is no evidence that the final thrombin yield is affected by varying the Ca in the range studied This could mean that any calcium even a trace could convert prothrombin to thrombin via the postulated 'intermediary' (p 83) and then be released again to convert another portion of the prothrombin

Effects of Traces of Calcium in Prothrombin Prepa rations Unless ion exchange resins are used it is extremely dif ficult to be sure that all traces of calcium are removed from any plasma fraction including prothrombin There is always the question of protein bound Ca and the possibility of some free Ca^{++} released in attaining the equilibria studied by Nordbö [352] We theorize there fore that the so called 'spontaneous' activation of prothrombin e g (1) in the experiments of Howell [233] and his pupil Cekada [71] and (2) in our own experience [156] with some of these and with Seegers' highly purified prothrombins are consistent with this ability of traces of Ca contaminant (and likewise traces of thromboplastin and co

factors) slowly but significantly to convert the prothrombin to thrombin by mechanisms which are identical in their essential nature with the processes going on in ordinary optimal activations

(6 J) THE QUESTION OF 'SPONTANEOUS' ACTIVATION OF PROTHROMBIN TO THROMBIN TRACE CONTAMINANTS? STABILITY OF PROTHROMBIN AND THROMBIN

Experiment 26 (1948) Table XXVIII

Purpose To investigate spontaneous' thrombin formation in highly purified (Seegers') prothrombin

Method This experiment was actually the chance outcome of keeping a solution of Seegers' purified prothrombin (Prep H cited [146]) for 9 months in the ordinary (4°C) refrigerator and re testing out of curiosity at the end of this period It was found to be active thrombin A dilution series (II) was prepared with the same amounts of calcium and tissue thromboplastin (not incubated) as had produced maximal activation of the original prothrombin solution 9 months earlier At that time such a dilution series had been tested and the data (I) were available for comparison

Results These are given in Table XXVIII They clearly show identity of the thrombic potencies at successive dilutions with minor divergence only at the highest dilutions which are always subject to some experimental error It is remarkable that as short a clotting time as 4 sec accurately measured with a stopwatch has real meaning as a measure of thrombic potency With such matching of serial dilutions it is an inescapable conviction that the two thrombins are indeed identical

Conclusion It must be concluded therefore that any spontaneous conversion of prothrombin to thrombin may very well be due to trace contaminants of calcium and other activators These trace amounts may be far too little to analyze Nevertheless given sufficient time they may complete the conversion to a thrombin of potency identical with that ordinarily obtained after a short incubation period with optimal calcium and other activators This obviously suggests that the calcium and other activators may be used over and over again in transforming the prothrombin bit by bit to the final end product From one point of view this type of reaction may be regarded as 'catalytic This idea would seem difficult to apply to so simple an ion as Ca^{++} were it not for an explanation on the basis of an 'intermediary complex such as we have argued for on the basis of a variety of other experiments

Additional Considerations It is valid to draw from the above experiment the following further conclusions

1) Prothrombin and thrombin are remarkably stable proteins in the absence of any factors which can alter them

2) Our purified system is particularly free from inhibitors It is possible to visualize a less pure system containing antithrombin for instance in which very slow conversion of prothrombin to thrombin

calcium optimum at this 8 mM level Above this level the most con sistent effects of increasing the Ca are the longer c t 's in the one minute test just as is the case (Experiment 23) in reducing the Ca conc below this optimum level In the 5 min test however there appears to be some slight improvement with increasing Ca

Conclusion It would seem that the main effect of varying the cal cium level in our thrombin forming mixtures is to modify the initial rate of thrombin formation Effects on the final thrombin yield are very questionable at Ca levels above the optimum and negligible (if any) at sub optimal Ca concentrations

Experiment 25 (1948) Table XXVII

Purpose To study the effects of varying amounts of Ca^{++} during prothrombin activation using Seegers' [431] 'purified' prothrombin in presence of tissue thromboplastin

Foreword This experiment of the author's was published in 1948 [156] In retrospect we were dealing with a very stable prothrombin undoubtedly containing proconvertin but very poor in AcG (hence the slow activation) The proper second phase controls were included

Method and Results are given in Table XXVII They demon strate 1) no activation (in 2 hrs) in the absence of added Ca 2) slower rate of activation if the Ca conc in the thrombic mixture is too low (2) or too high (4;5) but 3) no effect on the final (2 hr) throm bin yield i e identical (4 sec) minimal c t end points

Conclusions (Experiments 23 25) The cited experimental data permit the important conclusions that the rate of thrombin formation from prothrombin in the presence of adequate thromboplastin and co factors (proconvertin and AcG) is determined by the calcium con centration in the activating (T M) mixture Too little or too much calcium retards the thrombin formation and there is a definite Ca optimum at about 0 008 M in protein poor mixtures at pH 7 3 and 0 15 ionic strength However there is no evidence that the final thrombin yield is affected by varying the Ca in the range studied This could mean that any calcium even a trace could convert prothrombin to thrombin via the postulated 'intermediary' (p 83) and then be released again to convert another portion of the prothrombin

Effects of Traces of Calcium in Prothrombin Prepa rations Unless ion exchange resins are used it is extremely dif ficult to be sure that all traces of calcium are removed from any plasma fraction including prothrombin There is always the question of protein bound Ca and the possibility of some free Ca^{++} released in attaining the equilibria studied by Nordbö [352] We theorize there fore that the so called 'spontaneous' activation of prothrombin e g (1) in the experiments of Howell [233] and his pupil Cekada [71] and (2) in our own experience [156] with some of these and with Seegers' highly purified prothrombins are consistent with this ability of traces of Ca contaminant (and likewise traces of thromboplastin and co

5) While this prothrombin solution was unstable in the sense that it slowly changed to thrombin on simple keeping yet it was amazingly stable in the sense of yielding the same potency thrombin and in the same incubation time when activated by Ca and tissue thromboplastin (two different preparations) in tests two months apart

6) Calcium alone gave an identical end point and it must be conceded that this although requiring a 4 day incubation is just as complete a thrombin formation Hence it must be assumed that the 'purified' prothrombin still contains significant traces of thromboplastic and other necessary activators

Experiment 28 (1948) Table XXX

Purpose To study further the stability of prothrombin in the presence of Ca^{++} and thrombin

Method Since previous experiments had shown the very slow formation of thrombin on addition to highly purified (Seegers') prothrombin of Ca salt alone it was evident that such thrombin as was slowly formed would have every opportunity over several days to exert any possible effect on the still unaltered prothrombin Were there any such effect it would be expected to show up in the final (end point) clotting times of test samples removed at intervals and rapidly (within 1 hr) activated by addition of tissue thromboplastin

T.M.: 15 ml 0 35% Pro F (Dr Seegers preparation assaying 13 700 units per mg tyrosine N or 2450 units (2 stage) per ml of solution) + 5 ml borate buff + 1 ml 0 1 M $CaCl_2$

Test Series (A): 0 5 ml 0 5% B F + 0 25 ml diluted T M i e 2 ml + 0 25 ml buffer to serve as control for (B)

Test Series (B): 2 ml T M removed after stated periods and treated with 0 2 ml thromboplastin (various tissue preparations) with successive testing of 0 25 ml samples on 0 5 ml B F (0 5%) until reaching the minimal clotting time end point noted

Results These are given in Table XXX Note that in every case no matter what the age of the recalcified T M or which type of tissue thromboplastin was used the (B) series end points were all exactly 4 sec and identical with that reached sometime between the 3rd and 8th day in series (A)

Conclusion This experiment completely fails to demonstrate any destruction or other effect of thrombin co existing for days with its original (partly unchanged) prothrombin (cf [494]) These observations are important in ruling out possibilities of interfering reactions in the type of experiment relied upon for most of the data in the present investigations

(6 K) TESTING FOR (A) THROMBOPLASTIC AND (B) ANTI THROMBOPLASTIC ACTIVITY IN VARIOUS LIPOIDAL MATERIALS ESPECIALLY CEPHALIN AND SYNTHETIC PHOSPHATIDES AND THEIR CONTAMINANTS

Earlier Data (Table XXXI) Using T M containing Howell type prothrombin (cf Experiment 20 p 89) data were obtained by the

may be proceeding but with the thrombin being removed as fast as it is formed by the action of antithrombin Such a system would merely show a gradual loss of the ability of the remaining prothrombin to yield the original amount of thrombin in successive samplings tested after the conventional activation

3) At the dilutions studied viz about 1000-2000 two-stage pro thrombin (Seegers) units/ml the thrombin formed and in contact with the residual still unaltered prothrombin for weeks or months showed no evidence whatsoever either of (a) 'autocatalytic' acceleration of the prothrombin conversion or of (b) any destruction of the prothrombin by the thrombin (cf [494]) We have repeatedly mixed fully formed thrombin with a prothrombin activating system and failed to find any acceleration of the activation process with only one exception namely in the presence of platelets This exception will be studied further in connection with the platelet experiments (p 109)

4) Either minimal traces or significant small amounts of thrombin could be detected in all 8 highly purified prothrombins viz 7 made by Dr Seegers and 1 by ourselves which were studied very exhaustively in 1948 investigations [156] Some of these data bearing on stability questions will be reproduced as follows

Experiment 27 (1948) Table XXIX

Purpose To investigate the stability of prothrombin in solution

Reagents Pro B: One of Dr W H Seegers' very highly purified bovine prothrombin preparations kindly provided for us with the in formation that it contained 15 200 units/mg tyrosine N or over 2000 units/ml of the 0 2 % solution used in the present experiments Tpln A Rabbit brain thromboplastin (commercial Squibb s) Tpln D Ex tract made with saline from frozen dog brain T.M. 5 ml in borate buffer (p 83) pH 7 7 with additions as stated of 0 25 ml 0 1 M $CaCl_2$, with or without 0 25 ml thromboplastin Clotting tests at 25 ± 2°C on 0 5 ml 0 5% B F (p 55) + 0 25 ml T M after stated incubation periods up to 7 days

Results are given in Table XXIX

Discussion 1) Initial tests about 2 hrs after making up a 0 2% solution (in borate buffer) of the lyophilized prothrombin B showed a trace of active thrombin estimated to be less than 0 5% of the total potential thrombin yield (according to dilution tests given in the cited publication)

2) The relatively slow activations in these 1946 experiments we believe may very well be attributed to lack of sufficient proaccelerin

3) The 1 min tests chiefly represent active thrombin in the solu tion since very little thrombin will be formed in this very short period under the slow activation conditions noted in (2) The first test in series (3) therefore indicates a considerable increase in thrombin contaminant spontaneously appearing in the 2 months storage at 4°C in the icebox

4) Although the activation is much slower in (1) with Ca alone it finally (in 4 days) reaches a stable end point c t = 3 sec identical with that reached in a couple of hours in the other two series

Discussion (ref Table XXXII) The significant findings to date are:

1) Synthetic cephalins containing only saturated fatty acids e g (tests 2 3 4) 1 2 distearoyl 1 2 dipalmitoyl or 1 3 dimyristoyl phosphatidyl ethanolamines ('α' in the first two and β' type in the third) are devoid of thromboplastic activity This confirms earlier data by Grün and Limpächer [202] and Kabashima and Suzuki [263]

2) Efforts to synthesize cephalins containing unsaturated fatty acids are currently under way but not yet available for presentation in this thesis

3) A typical 'acetal' phosphatide but again with only saturated fatty acids viz stearal acetal phosphatidyl ethanolamine (test 5 see p 98) is inert in our thrombin forming test systems so is the corresponding palmital compound (test 8) except for an extremely doubtful ±

4) a myristal acetal phosphatidyl ethanolamine (test 6) which was 85 90% pure assayed quite inhibitory (anti thromboplastic) This looked like a possible lead toward the identification of a lipoid anti thromboplastin However when this synthetic compound was further purified it (test 7) lost the inhibitory properties

5) All efforts to run down the real inhibitor gave only the equivocal answer that it was residual in one or other trace fraction of the mother liquors (tests 9 12) None of the possible components which could actually be identified turned out to have significant effects in our test system and we are unable to suggest its true nature There was a similar trace of inhibitor in the supernatants (fraction I test 17) in preparing the palmital acetal P lipid Another trace contaminant (II) test 18 however gave only the negligible ± test Some of the trace fractions (tests 13 16) gave increased inhibition when added back to the purified phosphatide (No 7)

6) One possible lead resulted from these efforts namely that the trace contaminants (9 11 not 12) showing the most significant effects were mixtures of the less active ones Indeed we were able to demonstrate this (15 16) by actually mixing two of the less inhibitory fractions This enhanced inhibition by the mixtures suggests a possible synergism

Conclusions These new efforts to identify (a) thromboplastic lipid(s) and (b) antithromboplastic lipid(s) via the approach of synthetic organic chemistry are still very preliminary They do lead to the conclusions that

1) the ethanolamine base is not the primary determinant of thromboplastic activity

2) known cephalins containing saturated fatty acids only are inactive whether in the α or β form

3) acetal phosphatides do not have either thromboplastic or anti thromboplastic activity associated with their acetal structure

4) Anything more than this is speculative at the present time Thus

5) it is merely a tentative hypothesis but not yet superseded that the physiological thromboplastic lipid must be either (a) a true cephalin of some specific type (? containing unsaturated fatty acids) or (b) a contaminant or possibly (c) some metabolic by product of the known cephalin group of phosphatides

Hence this thesis will rest with the current status of this field of

author in 1943 on four fractions obtained by Dr J Folch of the Rockefeller Institute N Y in the re purification of brain cephalin [168] Following brief preliminary mention [138] we included these data in the publication of the Transactions of the Second Conference on Blood Clotting and Allied Disorders of the Josiah Macy Jr Foundation [144] They are reproduced in Table XXXI Data concerning fractions the percentage referring to yield in gm per 100 gm original 'cephalin' mixture V = phosphatidyl ethanolamine (15%); III = phosphatidyl serine (27%) I - inositol phosphatide (22%); IV - unidentified mixture (8%) In personal communications Dr Folch stated that III and I could contain about 5 10% of V (true cephalin) and IV 'may very well turn out to be either identical with or closely related to phosphatidyl ethanolamine ' Thus the biochemical criteria were not fully adequate for a definite correlation with the thromboplastic activity qualitatively demonstrated by our very sensitive test which as shown in the table detected such activity in all four fractions although none of them was as potent as a weak buffered suspension of cat brain thromboplastin Moreover all the preparations supplied were dried and brownish from months of exposure to the atmosphere and must have lost a considerable amount of their thromboplastic potency Inconclusive as they are these tests nevertheless add some evidence to indicate that true cephalin is a potent thromboplastic phospholipid Any other conclusion will need further advance in the field of phospholipid chemistry

Current (1956) Data (Table XXXII) For the past few years the author has been using the preceding test system for the thromboplastic and antithromboplastic (anticephalin) assay of a number of synthetic phosphatides These agents have been prepared and chemically studied by colleagues Drs C E Anderson and C L Yarbro of the Biochemistry Department at the University of North Carolina A report to the 1953 annual meeting of the North Carolina Academy of Science recorded the finding of neither such activity in the testing of an acetal phosphatide (test 5 Table XXXII) Tests on other current materials are listed as to results in Table XXXII

Method Our two stage (Method III) technique as previously described (p 59) Several dog eluates' (p 71) were prepared for use in these studies The AcG (1:8) see p 58 was a single batch as was the 'standard' (dog brain) cephalin (p 71) The 'unknowns' were supplied to us identified only by a code number and the descriptive data given to us by our biochemical colleagues only at the (temporary) conclusion of these investigations The following criteria were used for degree (+ or) of activity

(a) ++++ end point clotting times of < 20 30 sec (at 25 ± 3°C) equivalent to > 10γ/ml obtained in incubation periods of < 10 30 min

(b) ±: slight enhancement of the std ceph but no significant thromboplastic activity alone

(c) - -: end point clotting times in 30 min with std ceph (50γ/ml) co addition of over 5 min i e essentially complete inhibition

(d) : end point (½ hr incub) c t of 3 5 min

(e) - end point (½ hr incub) c t of 2 3 min

(f) : end point (½ hr incub) c t of 1-2 min (<0 1γ)

tion With the amounts of agents tested in this particular experiment the heparin proved completely inhibitory to the cephalin (test 2) and practically so to the isolated phospholipid fraction (test 4) from the thromboplastin Distinctly different was the result of test 6 in which the heparin showed no effect upon the tissue thromboplastin except in the 1 min test Trypsin (see p 25) was also a good thrombop astic agent in these experiments (7) and resembled tissue thromboplastin in the very minor and early stage inhibition by heparin

Even though the thromboplastin preparation (5) had been kept (at 4°C) for 3 days pending analyses and preparation of (3) it was distinctly more potent than its equivalent of the extracted P lipids

Experiment 30 (1941) Table XXXIV

Purpose To demonstrate the significance of a heparin cofactor in its inhibitory effects during prothrombin conversion to thrombin [151a]

Method Similar technique to the foregoing

Reagents Pro: Howell type prothrombin (p 91) Tpln: Glass wool filtered saline suspension of frozen dog brain Ca: 0 1N $CaCl_2$ Hep 5 Toronto units/ml diluted in saline from a Connaught Lab (Toronto) preparation kindly supplied through the courtesy of Dr C H Best Stock solution in saline at pH 7 5 0 1% : 1 mg dry wt represented 110 Toronto units Cofactor: This agent was a crude albumin obtained by precipitating citrated dog plasma between 50 and 100 percent saturation with $(NH_4)_2SO_4$ dialyzing away excess of salt and obtaining a final solution in 0 9% NaCl at pH 7 5 T.M. 4 ml Pro + 5 ml cofactor (or saline in (1)) + 0 5 ml hep + 0 25 ml tpln + 0 25 ml Ca Incubated at 7 5°C to avoid any deterioration Clotting tests Respective T M s (0 5 ml) added to 1 ml fibrinogen (p 82) + 0 5 ml of saline (tests 1 5) or saline mixture containing amounts of hep cofactor or both required to make tests 6 7 8 equivalent in the final clotting mixtures to 2 3 4 respectively Tested at 38°C Tests 5 6 7 8 were made with T M (1) at maximal (60 min) activation

Results See Table XXIV Note the following

Discussion (a) The chosen amount of heparin alone had a negligible effect on the fully formed thrombin (6) but did inhibit thrombin formation (2) in the early phases

(b) This particular cofactor (albumin) was very slightly inhibitory to the 1 hr T M (1) in test 7 and to the later phases of T M (3) It was however slightly helpful to the earlier phases of activation in (3) In other experiments of a similar nature we have found either a little inhibition or a little aid in this early phase differing with various albumin preparations

(c) The combination of hep + cofactor (4) was completely inhibitory to thrombin formation in tests extending over 2 hrs This is the more significant because the test (8) on fully formed thrombin showed very little in the way of antithrombic action

Conclusion A cofactor present in crude albumin fractions is

knowledge and continue to identify the thromboplastic phospholipid(s) as cephalin(s) for want of a better working hypothesis

Addendum Dr K M Brinkhous kindly supplied us with a few milligrams of Dr E Baer's (Connaught Labs Toronto) synthetic preparation [28 29] of 1,2 dimyristoyl-phosphatidyl α ethanolamine ('X') with which our Pathology Department colleagues [282] had reported a *feeble thromboplastic action In our routine tests a thrombic mixture* which gave a minimal clotting time of a negligible 22 minutes (over 1 hr incubation) was able to give only a 6 min c t with 2 0 ml of 0 1% suspension of 'X' and barely improved to about 4 min c t with 2 0 mg of 'X' (per 5 ml T M) These are negligible results and point only to the most minute trace of something that can hardly be regarded as thromboplastic We must conclude therefore that this compound like our own and others [202 263] is essentially devoid of true thromboplastic properties

(6 L) ACTIONS OF HEPARIN WITH SPECIAL REFERENCE TO THE THROMBIN-FORMING REACTIONS

Experiment 29 (1939) *Table XXXIII*

Purpose To study effects of heparin on the formation of thrombin from recalcified prothrombin in the presence of various thromboplastic agents [131]

Method The usual two stage technique similar to previous experiments T.M.: 4 ml Pro (Howell-type p 91) + 0 5 ml $CaCl_2$ (0 05 m eq) + 0 25 thromboplastic agent (varied see below) + 0 25 dist water or heparin (0 5 mg equiv to 55 Toronto units)

Thromboplastic Agents

1) brain cephalin (p 71) 100γ per 5 ml T M

2) tpln. Q. a rabbit brain thromboplastin (dried preparation) prepared and kindly supplied by Dr A J Quick The present solution analyzed Protein 104 mg %; Total P lipids 11 mg % incl 4 6 mg cephalin

3) brain P lipid Quick's thromboplastin was extracted with alcohol ether (3 1) the acetone insoluble P lipids being recovered in petroleum ether dried and prepared in a 1:10 000 aqueous solution The amount added in tests 3 4 represented the equivalent in phospholipid of the original tpln Q used in tests 5 6

4) crystalline trypsin 125γ a preparation kindly supplied by Dr M Kunitz of the Rockefeller Institute Princeton N J

The T M mixtures were incubated at a cool temperature 15°C in order to minimize any instability but clotting tests after the stated incubation periods were made at 38°C with 0 5 ml T M + 1 ml fibrinogen (p 82)

Results These are given in Table XXXIII

Data and Discussion Any cofactor (see later) which the heparin may need must have come from the Howell-type prothrombin prepara

Experiment 31 (1948) Table XXXV

Purpose To demonstrate that the mode of action of heparin in de laying the conversion of prothrombin to thrombin is anti thrombo plastic' in nature

Foreword In the previously cited 1941 publication [151a] the au thor (with Dr A J Glazko) activated Howell prothrombin with Ca and tissue thromboplastin (in 1/1 1/2 1/32 dilutions) with and without heparin The significant observation in the heparin experiments was that the rate of thrombin formation was delayed by the heparin the more the weaker the thromboplastin The final thrombin yields (end point c t 's) were essentially similar however

Another experiment [156] performed about 1948 with one of Seegers' purified fibrinogens essentially confirmed the earlier find ing and was especially convincing because of the adequate controls This will be reproduced as follows:

Method Two stage testing of prothrombin activation by method essentially similar to preceding experiments

T.M.: 4 ml Pro. E (ref [156]) + 0 5 ml borate buffer (p 83) in control (1) or 0 5 ml heparin (100 units) in series (2) + 0 25 ml tpln A (rabbit brain Squibb = 0 25%) + 0 25 ml 0 1M $CaCl_2$ 25 ± 2°C

Clotting-tests: (1a) 0 25 T M (1) + 0 25 buff + 0 5 B F 1" (p 55)
(1b) 0 25 T M (1) + 0 25 hep (10 unit) + 0 5 B F 1"
(2) 0 25 T M (2) + 0 25 buff + 0 5 B F 1"

Results These are shown in Table XXXV

Discussion The data show:

1) complete activation to 4 sec end point in 8 hrs in (1a) with complete stability of thrombin for a week thereafter

2) some inhibition of the fully formed thrombin by the heparin as shown in (1b) This is very slight a mere ½ sec (real) difference after completion of the T M activation The smaller amounts of thrombin in the earlier phases of activation are more significantly in hibited (delayed) by the heparin however

3) inhibition of thrombin formation in the presence of the heparin is clearly shown by comparing (2) and its control (1b)

4) However the final end point although it took a week to attain is identical (4½) in the (2) and (1b) series

Conclusion Comparing this result with effects of a minor reduc tion in amount of thromboplastin (Table XXIII also reproduced from the 1948 paper [156]) the heparin inhibition of thrombin formation may be regarded as due to an interference with the action of the throm boplastin Using a potent tissue thromboplastin the inhibition refers to a delay in rate of thrombin formation with no effect on the final thrombin yield

Considerations Regarding Modes of Action of Heparin It should be emphasized that in these experiments no 'cofactor' was added and was unlikely to be present as a contaminant of the highly purified prothrombin Further the heparin was added in relatively

therefore able very greatly to increase the inhibitory effect of heparin during thrombin formation even when the combined effects after full thrombin formation are almost negligible These data support the view that cofactor is needed for the action of heparin in preventing prothrom bin activation and this phenomenon is distinct from the established [242 382] antithrombic action of heparin + cofactor which is exerted upon fully formed thrombin

Addenda (I) Brinkhous Smith Warner & Seegers [67] in 1939 presented the first evidence that a serum cofactor is needed for hepa rin to prevent the conversion of prothrombin to thrombin Their data may be briefly reviewed One of Seegers' [435] early purified pro thrombin preparations yielded 363 two stage units after 2 hrs activa tion by Ca and rabbit brain thromboplastin and practically as much (343 units) when heparin was also added With heparin plus serum (co factor) thrombin formation was blocked and almost all the prothrom bin remained unchanged for two hours With the serum alone all but 52 units of prothrombin were converted into thrombin in the same time period This experiment shows incidentally that serum antithrombin acts only on fully formed thrombin By and large this experiment proved the new observational fact It is certain however that the two hour incubation period masks any effect which the heparin and serum alone might have on the rate of thrombin formation Because of this doubt we performed the 1941 experiment [151a] cited above which we believe established the validity of the Iowa workers' conclusion beyond any reasonable question besides adding some new information as to what is going on during the activation period

(II) The 'antithrombic' action of heparin + cofactor was first ob served in the original experiments of Howell & Holt [242] Quick [382] found the cofactor to be associated with the albumin rather than the globulin in crude plasma fractions Astrup [20] has reviewed the hep arin studies made in the Carlsberg Foundation Laboratories (Copen hagen) including a 1939 confirmation of the Iowa discovery (above) and an important suggestion [21 22] that the co factor necessary for turning heparin into an antithrombin which we have called 'thrombin coinhibitor ' is a far more delicate substance than hitherto assumed It disappears from plasma during clotting and is therefore not found in serum whether prepared by addition of thrombin by spontaneous clot ting or by recalcification It is further inactivated completely by heat ing to 56°C for 5 minutes

(III) Since the topic of this thesis is primarily designed to explore the clotting functions of certain lipids (e g cephalin) and complex ma terials (thromboplastin) in which they occur experiments with heparin are relevant when they deal with antithromboplastic inhibitions of the thrombin forming reaction *To go into antithrombic actions however* would unduly extend our enquiry We shall therefore omit some other wise valuable experiments e g (1) on heparin + cofactor as antithrom bin [131] and (2) some unpublished data confirmatory of Astrup & Darling [22] which show by a different method that serum is devoid of heparin cofactor but because of progressive removal of added thrombin by the classical serum antithrombin (Astrup [20]) this thrombin becomes so weak in the serum mixtures that it is now very sensitive to the immediate inhibitory action of heparin in an effect which requires no cofactor

3) added calcium and even better Ca + cephalin greatly enhance the effects of trypsin Hence

4) the author's (with Mrs Betty Nims Erickson's) 1939 conclusion [149] that trypsin is not a thromboplastic agent in its own right (as Eagle & Harris [112] erroneously concluded) but merely makes Ca^{++} and 'free' cephalin available for the prothrombin activation presum ably by releasing them from otherwise inert protein combinations Mrs Erickson and the author were never quite able to provide the con vincing proof for this attractive theory and such is still unavailable However a 1948 experiment with B L Travis and E B Gerheim (graduate students) ref [156] did seem to add some new evidence on the need for both calcium and thromboplastin in order to obtain the actions of trypsin in thrombin formation These experiments there fore will be recapitulated

Experiment 32 (1948) Figure 17

Purpose To demonstrate the need for Ca^{++} and thromboplastin in the trypsin activation of purified prothrombin

Reagents Pro, C (ref [156]) purified by Dr W H Seegers 0 3% solution Tryp Crystalline trypsin prepared by Dr M Kunitz [323] 40 units/ml Tpln, A; Squibb s rabbit brain thromboplastin 0 25% suspension Ca: 0 1 M $CaCl_2$ T.M. (thrombic mixtures):

(I) 4 ml Pro + 0 75 ml borate buffer (p 83) + 0 25 ml Ca;
(II) 4 ml Pro + 0 25 ml buff + 0 5 ml tryp + 0 25 ml Ca;
(III) 4 ml Pro + 0 5 ml buff + 0 25 ml tpln A + 0 25 ml Ca;
(IV) 4 ml Pro + 0 5 ml tryp + 0 25 ml tpln A + 0 25 ml Ca

Clotting tests at 25°C on 0 5 ml B F (p 55) + 0 25 ml T M after successive incubation times (i t)

Thrombin percentages were obtained by comparing the observed clotting times with a dilution series of maximally activated (24 hr) T M III The dilution data are plotted as 1000/c t (sec) in the dotted line of Figure 17

Results are depicted graphically in Figure 17 originally published [142] in the Ann N Y Acad Sci 1948 Note the following:

1) Ca alone produces a very slow activation complete in 50 hrs

2) Trypsin with Ca is not significantly better

3) Ca + tpln gives adequate prothrombin conversion but is rather slow under the particular experimental conditions (? lack of AcG) be ing about 50% complete in 4 hrs and 100% in about 24 hrs

4) Trypsin significantly improves the Ca + tpln causing the reac tion to be over 90% complete within 1 hr and delayed (16 18 hrs) only in the penultimate stages

Conclusion Trypsin does not act alone or with calcium only but requires a source of thromboplastic agent With this further addition the enzyme has a significant effect in accelerating the thrombin forming reactions

high concentration It may therefore be reasonably concluded that these are some special experimental facts pertaining to high concentrations of heparin (itself highly purified) They may have no real connection with a more physiological system in which a much smaller amount of heparin inhibits thrombin formation by acting in conjunction with a plasma cofactor However similar results were obtained with physiological amounts of heparin in Experiment 47 p 120 The physiological significance of heparin has been reviewed on pp 30 31 Our researches have added a little to the understanding particularly of one of its possible modes of action namely to prevent prothrombin activation by functioning as an 'anti thromboplastin ' Study of this phenomenon in turn adds to the general knowledge of the thromboplastic mechanism and its very fundamental role in physiological thrombin formation

We are endeavoring in this thesis to draw only those conclusions that are well supported by experimental fact and wish to avoid theoretical speculation as much as possible In the heparin problems we are still on insecure ground and the following suggestions are very tentative but may be worth presenting as a possible lead for future investigations Heparin (with its sulfuric acid moities) and cephalin (with its phosphoric acid groups) are both acidic substances known capable of combining with proteins and basic protein split products such as protamine (Chargaff & Olson [80]) May it not be possible therefore that the more acidic heparin can block or displace the essential thromboplastic phosphatide from certain protein combinations which are normally essential for the P lipid to participate in the process of thrombin formation (prothrombin activation)? May it not further be postulated that the cephalin is thereby deviated to some other protein say the cofactor altered (? made receptive) by the action of heparin? This could explain the 'antithromboplastic' action of heparin The 'antithrombic' action in somewhat similar manner could be a deviation of thrombin to some cofactor + heparin complex (Astrup & Darling's [22] 'thrombin inhibitor) which according to Fitzgerald & Waugh (cited p 31) functions as a somewhat special type of antithrombin

(6 M) THROMBOPLASTIC ENZYMES IN RELATION TO THROMBIN FORMATION BY THROMBOPLASTIC LIPOIDS AND (?) LIPOPROTEINS

Foreword 1939 Data The thromboplastic' action of crystalline pancreatic <u>trypsin</u> was briefly reviewed on p 25 and experimental evidence of this phenomenon was incidentally included in Experiment 4 (p 66) and Table V (on completely platelet free plasma) and in Experiment 29 (p 100) and Table XXXIII (on heparinized thrombin forming systems in which trypsin was substituted for tissue thromboplastin) much as Eagle (cited p 25) had substituted it for Ca + platelets We shall not cite the experimental details but merely refer to the conclusions of our early work [150 149 136] viz

1) trypsin can clot oxalated or citrated plasma and activate (Howell type) prothrombin even in the absence of added calcium and cephalin

2) sufficient excess of the decalcifying anticoagulants can prevent these actions of trypsin

Addendum These and other studies in the cited paper [156] give some new insight into the possible significance of 'trace contaminants' in even the best modern prothrombin preparations It is perhaps unfortunate that Dr Seegers has never seen fit to report on Micro Calcium (spectroscopic) and P lipid analyses of his prothrombin prep arations but merely to assert (with reasonable pride) their 'purity on the basis of pot ncy per weight of protein (or tyrosine N)

(6 N) PLASMATIC THROMBOPLASTIC COMPONENTS CONCERNED WITH THROMBIN FORMATION PTC AND AHF

Because thromboplastic function particularly of platelets is be lieved to be associated with a thromboplastin generating reaction (pp 46 et seq) in which PTC AHF and probably other factors are involved the following PTC and AHF assays were performed:

Experiment 34 (1956) Table XXXVII

Purpose To test for PTC and AHF in (I) dialyzed eluate (p 81) (II) Howell type prothrombin (p 91) and (III) Dr R H Wagner s [488] 'purified' AHG (antihemophilic globulin p 77) using (IV) a normal human plasma (D C F) as the factor control

Method Uses the specific one stage prothrombin assay (p 57) to test the improvement over saline (substrate control 0) of the pro thrombin consumption of recalcified substrate plasmas (R J PTC deficient [294]; W B AHF deficient [152] human clinical cases)

Test 0 2 ml substrate + 0 05 ml additive are recalcified with 0 2 ml 0 025 M $CaCl_2$ After 1 hr in the case of the AHF tests and after ½ hr in the case of the PTC tests (see Discussion) 0 05 ml of 0 1 M sod oxalate is added to check further prothrombin utilization 1 stage prothrombin assays are performed on (1) the original mixtures and (2) the serum after oxalation and centrifugation (to remove clots) Amounts of additives are specified in Table XXXVII

Results Table XXXVII gives the experimental details and test findings Using reference curves similar to those illustrated in Fig ure 16 (I) but obtained from human (instead of dog) standard normal plasma the 1 stage clotting times (c t at 37°C) are converted into the cited prothrombin (Pro) values expressed as percent of these normal standards

$$\text{Prothrombin consumption } (\%) = \frac{\text{original pro } (\%) \quad \text{serum pro } (\%)}{\text{original pro } (\%)} \times 100$$

Discussion The PTC control (0) showed no clotting within 30 min (37°C) whereas all the additives caused clotting well within that period At the time of these tests our substrate subject (R J) did show some consumption of prothrombin but gave significant assays when the incu bations were restricted to ½ hr R J also presented another somewhat complicating problem in that he had a small amount of inhibitor (anti PTC [294 152]) This made his plasma less sensitive in our assays but still serviceable as the results indicate

The AHF control showed a trace of clot in 22 min whereas the

Experiment 33 (1948) Table XXXVI

Purpose To study further the role of trypsin in systems of activating purified thrombin

Method Essentially similar to Experiment 32 including computation of 'percentages' (of maximal activation)

Reagents Pro. D (ref [156]) another of Dr Seegers' purified bovine prothrombins 0 2% solution Tpln. D saline suspension (decanted) from frozen dog brain (p 101) 0 1% Two amounts were tested Tryp Same crystalline trypsin 40 units/ml T.M.'s all contain 4 ml Pro D + stated amounts of activators + borate buff to total 5 ml vol
Clotting tests: 0 5 ml B F (1%) + 0 25 ml T M incubated as stated 25°C

Results are given as 'percentage' activation in Table XXXVI

Discussion
1) With Ca only activation is very slow and measurable only after 1-3 hr but is complete (100%) in 48 hrs
2) With trypsin alone activation is slow and poor with best thrombin yield (20%) in 6 hrs The decline thereafter points to a possible thrombolytic action of trypsin
3) With Ca + trypsin a fairly good activation is noted with 100% completion in 3 hrs
4) With Ca + tpln at the higher concentration gave good thrombin formation complete in 30 min This was used as the reference standard
5) With Ca + tpln at the weaker (1/3) concentration was much poorer and required as long as (I) viz 48 hrs to reach 100%
6) With Ca + tpln (1/3 str) + trypsin the activation was much improved and complete in 3 hrs However this is only a little better in the earlier stages than in (III)

Conclusions (Experiments 32 33) Both the cited experiments prove that the enhancing (accelerating) effect of trypsin upon the conversion of 'purified prothrombin to thrombin depends upon the presence of other activators namely calcium and thromboplastin It is particularly when the calcium is adequate and the thromboplastin suboptimal that trypsin (experimentally) shows its most marked effects It may be further concluded with some slight reservations because of the lack (and insuperable difficulty in obtaining) exact evidence that these highly purified prothrombins still contain traces of activator impurities The differences in the results of the two experiments while slight may be significant suggestive evidence that (1) Pro C (Experiment 32) contains insufficient trace of thromboplastin for the trypsin to add anything to the very minor effect (by and large) of added calcium whereas (2) *Pro D (Experiment 33) contains sufficient traces* of some thromboplastin impurity for trypsin to exert a very definite effect with Ca The further addition of the weak (1/3 str) tpln D does not add much to the postulated thromboplastin contaminant so that its inclusion (series VI) gives little better activation than in (III)

imid buff sal before use Its proteolytic activity was tested in Experiment 44 (p 182) Plat 10x washed frozen and thawed dog platelets prepared October 29 1952 (!) and frozen stored at 20°C T.M. 5 ml mixtures in imidazole buffered saline containing 0 1 ml eluate 0 1 ml AcG (1 5 dil) + stated amounts of 'thromboplastic' additives + 0 5 ml 0 02M $CaCl_2$
Clotting tests 0 2 ml fibrinogen (dog) + 0 2 ml T M after successive incubation periods 27°C

Results These are given in Table XXXVIII and show the following
1) with platelets as the only thromboplastic additive thrombin formation is weak but unequivocal and yields a 30 3 c t end point in 30 min corresponding to about 35% activation of the prothrombin
2) with AHG a marked potentiation is seen in the 5 min and 10 min tests The trace of fibrinogen in the AHF preparation caused a small clot in T M (2) in about 4 min This was removed and the mixture remained clear thereafter It is possibly the adsorption of a little thrombin on this fibrin (removed) which explains the somewhat longer c t 's in the later tests of (2) The end point is at about 30 min as in (1)
3) a control with AHG only (no platelets) is very poorly activated but does yield a 164 2 c t in 30 min
4) with trypsin the potentiation is very marked Even after 1 min incubation a 155 5 c t was obtained whereas the other series (1) (2) did not give a clot in the 1 min incubate in over 2 hrs Moreover the end point (35 7) was definitely hastened to a 10 min incubation period
5) trypsin alone is very feebly thromboplastic with a trace of thrombin formation still continuing after incubation for 1 hr (445 →)
6) trypsin enhanced the minor effect of AHG (in absence of platelets) in 10 20 min incubations but the 30 min end point (172 6) was if anything a very little inferior to (3) Here again a trace of fibrinogen (less than in (2)) required removal after 7 min

Conclusion Antihemophilic globulin (AHG) is clearly able to 'potentiate the weak thromboplastic activity of an old (over 3½ years!) well washed (x10) frozen stored (20°C) dog platelet suspension A trypsin solution (from a stock 2% sol in 50% glycerol + borate buffer) which had been preserved in the ordinary refrigerator for over 11 years was an even more striking 'potentiator' of the platelet activity The actions of AHG trypsin individually and in combination effect very little thrombin formation in our eluate mixtures containing prothrombin proconvertin PTC and added AcG + Ca but no added thromboplastin

Experiment 36 (1956) Table XXXIX.

Purpose To study the potentiation of platelet thromboplastin by (1) fibrinolysin and by (2) weak thrombin

Method This experiment was performed on the same day (May 24 1956) by the same technique as Experiment 35 (p 108)

Reagents The salts including 0 02M $CaCl_2$ eluate (dialyzed) AcG (1 5) and platelet preparation were the same and used in the same amounts as in Experiment 35

additives caused good clotting in less than 220 sec The 10% (1 hr) prothrombin consumption in the AHF control (0) is a severe deficiency All the materials tested showed the presence of both PTC and AHF in varying degree

Conclusions Our 'prothrombin' reagents both (I) eluate and (II) Howell type prothrombin are quite rich in PTC They also con tain significant amounts of AHF about the same in the eluate and in the Howell prothrombin In the case of the eluate this must mean that some AHG is co adsorbed on the $BaSO_4$ and resists the distilled water washings (p 71) The traces of PTC in the 'purified' AHG may be due to the use of $Al(OH)_3$ in the preliminary plasma adsorption This is not quite as effective as $BaSO_4$ in removing PTC That the detected amounts of PTC were only traces is evident from considera tion of the concentration of the AHG tested (1%) In the AHC assays a 0 001% AHC addition gave an almost identical correction as 1 10 normal plasma which indicates about a hundredfold concentration of AHF in the stock 1% AHG solution

Addendum Later another PTC deficient case (W S) presented the opportunity for re testing of the Howell prothrombin and the AHG preparation This substrate in the control (0) showed a 37% pro thrombin consumption in ½ hr at 37°C A 1 10 normal plasma (D C F p 107) corrected this to 80% whereas the AHG (1%) gave 96% and the Howell prothrombin 100% This is excellent confirmation of the Ex periment 34 data on an inhibitor free substrate It definitely confirms the trace of PTC contaminant in the AHF preparation

7 TESTING OF CLOTTING FACTORS IN BLOOD PLATELETS

The clotting factors in blood platelets are reviewed on pp 43 51 The following experiments contain some new information besides con firming some previously established facts Tests on the enzyme and anti enzyme preparations used will be deferred to a separate section (p 117) following the thrombin formation experiments

(7 A) THROMBOPLASTIC ACTION OF PLATELETS POTENTIATION BY (a) AHG and (b) TRYPSIN

Experiment 35 (1956) Table XXXVIII

Purpose To study the thromboplastic action of platelets and the modifying effects of added (a) antihemophilic globulin (AHG) and (b) trypsin

Method The two stage procedure on dialyzed eluate (see Table VI) as repeatedly used in previous experiments

Reagents Eluate (No 127) dialyzed as previously described (p 81) AHC Dr R H Wagner's [88] 'purified' preparation of May 9 1956 described on p 77 Tryp : 2% trypsin (Fairchild Bros & Foster N Y) in glycerol borate buffer (equal vols) prepared Jan 5 1945 (I) and stored in refrigerator at 4°C Dil 1 100 with

ments of this era [476] which sought to relate platelet functions in the thrombin forming system with proteolytic enzymes and various anti proteases will be reproduced in the following

Reagents Some special testings of the enzyme and anti enzyme preparations will be deferred to a later section (p 117)

Borate Buffer 11 25 g H_3BO_3 + 4 g $Na_2B_4O_7$ 12 H_2O + 2 25 g NaCl + dist water to 1 liter pH (glass electrode) 7 7 + 7 75 specific resist ance 170 ohms (at 21°C) effective ionic strength 0 055

Fibrinogen B F 1% borate buff sol of $BaSO_4$ adsorbed bovine fibrinogen (Armour's p 55)

Thrombins (a) Thr bovine thrombin (Upjohn s p 56) courtesy of Dr J T Correll A 100 units/ml 'stock sol was prepared in borate buff (b) Thr * A special type of thrombin was prepared for one of the experiments and will be described in that connection (p 112)

Prothrombins Pro Several bovine prothrombins were made in our own laboratories following the directions of Seegers et al [431] However Dr Seegers kindly supplied us with a number of his own highly purified preparations Our [156] extensive studies using these products were reported in 1948 Excellent as Dr Seegers preparations were they did show evidence of trace impurities the control testing for which was an important part of our studies One of these trace contaminants was active thrombin In the tests to be cited it should be mentioned that no prothrombin solution was used (with rare deliberate exceptions which are stated) unless a preliminary test (0 5 ml B F + 0 25 ml Pro) yielded no trace of clotting in longer than 30 min Many of the experiments in the 1948 publication [156] were made before the establishment of our modern knowledge of the accel erator or co factors (proaccelerin or AcG syn factor V labile factor and proconvertin or SPCA syn factor VII stable factor etc) In the later experiments particularly those about to be cited [476] AcG was always added to our thrombin forming systems The only exception was when studying the role of this particular factor

AcG of bovine serum type was prepared and kindly supplied by Drs Ware and Seegers A 0 1% sol in borate buff showed traces of prothrombin and very minute amounts of active thrombin but these were considered to be non significant in the experiments cited It had no thromboplastic effects (author s tests)

Thromboplastins Tpln. A was prepared by ourselves from acetone dried dog brain a borate buffer suspension of which (0 25%) showed a high degree of thromboplastic activity Tpln. B Schieffelin & Co s Soluplastin (p 56) supplied through the courtesy of Dr E W Blanchard was used in some tests

Ca 0 5 M $CaCl_2$

Fibrinolysin Lysin: a 'purified enzyme preparation from beef serum [302] supplied through the courtesy of Dr E C Loomis (Parke Davis & Co Detroit) 1% sol in borate buffer was prepared immedi ately before use In later work we used our own dog fibrinolysin [291]

Antifibrinolysin Afln a purified' fraction from beef serum also supplied by Dr Loomis [303] in November 1947 A 0 4% extract was made with borate buffer and centrifuged from a considerable residue of insoluble material

Trypsin Tryp. A Crystalline trypsin (from pancreas) prepared

Lysin A 43 month old preparation of (dog) serum fibrinolysin de scribed on p 118 The 1 40 borate buffer dilution of this lysin was taken from frozen storage (20°C) thawed and used in the experiment i e 0 1 ml per 5 ml T M

Thr Bovine thrombin (Upjohn's p 56) 0 1 units/ml in buff sa line freshly prepared clotted an equal (0 2 ml) vol of test fibrinogen (dog) in 6 min At the 1 50 dilution in T M (7) therefore there was not more than 0 002 of a thrombin unit per ml This extremely small amount could not possibly add its own direct effect to the observed clotting times The control (9) without platelets or enzymes serves for both Experiments 36 and 35

Results are given in Table XXXIX and show the following Compared with (a) the negligible traces of activation (9) in the absence of platelets and enzymes (which are practically without effect alone) and with (b) the test (1) of Experiment 35 with platelets alone both the aged fibrinolysin (8) and the very weak thrombin (7) give evidence of a significant potentiation of the platelet thromboplastic action That this is not more marked (as in Experiment 37 p 112 for instance) is probably due to our use of unnecessarily weak additives Nevertheless the considerably shorter c t 's in the 5 min test are quite meaningful

Conclusion Fibrinolysin and thrombin therefore are two other agents with which we are able to demonstrate a 'potentiation' of the thromboplastic action of platelets Considering the age (over 3½ years) of the platelet preparation a remarkable stability of the platelet thromboplastin is manifest

Older Experiments In 1936 the author [126] performed some clotting experiments with platelets extracts of the cornea and lens of the eye (chosen because of absence of a blood supply) and prothrombin activated by Ca and cephalin In retrospect these data do not seem to merit inclusion among the much more definitive types of experimentation in the present thesis except for mention of the fact that they led to the formulation of a cephalin availability theory (see later) and the suggestion that prothrombin (the protein factor of the coagulant) may occur in platelets cornea and lens as the possible result of diffusion from the blood stream via the lymph and tissue fluids in the case of the eye tissues

In the next decade only a few experiments were made with platelets (substituting for cephalin) in the activation of Howell type prothrombin in the presence of calcium These did not seem to offer anything new Eagle & Harris' [112] experiments (1937) which suggested that trypsin could coagulate blood by the same effect as the physiological system Ca plus platelets (or Ca plus tissue extracts) inspired the present writer to an extensive series of studies with trypsin and later other proteolytic enzymes including fibrinolysin (plasmin or tryptase ref [143]) in an endeavor to learn more about their possible relations in the blood clotting system

In the period 1947-1949 and subsequently many experimental testings of platelets in artificial clotting systems were made by the author and his graduate students notably B L Travis Some of the experi

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same prothrombin preparation in borate buffer which had been al lowed to stand with $CaCl_2$ for several weeks in the refrigerator (4°C) Tests showed that it was completely converted into thrombin since incubation with thromboplastin and AcG gave no further enhancement of its clotting potency Such a preparation seemed well chosen to avoid the introduction of any factors except the thrombin unless per chance there are some unknown factors in the (recalcified) prothrom bin itself With these unsupported reservations we must conclude that weak thrombin can act as a remarkable potentiator of platelet thromboplastin Since AcG is added this would not appear to be due to the known effect [492] of thrombin in converting proaccelerin to accelerin Attempts were made to run controls without platelets These showed only insignificant shortening of the clotting times in the parallel test (O) without activators (except for the Ca) This cited test (O) merely showed the trace of thrombin in the freshly prepared prothrombin solution and this could not be correlated with the weak thromboplastic action of the platelets shown in (1) The added thr * (p 111) (diluted) clotted a test fibrinogen in 750 sec when freshly prepared but rapidly lost potency on standing in glass

The fibrinolysin test (3) showed a potentiation of the platelet *thromboplastic effect qualitatively resembling that of thrombin* and even quantitatively similar in this particular experiment It is diffi cult to test a fibrinolysin for traces of thrombin because the enzyme lyses the test fibrinogen before it has a chance to clot with a very weak thrombin We did perform this control test however in the presence of antiproteases (esp antifibrinolysin) which seemed to rule out this possibility It is not possible therefore to deduce a common mode of action for these two dissimilar agents (thrombin and fibrinoly sin) with any factual knowledge currently available

Conclusion We present the experimental facts therefore which prima facie indicate that (1) platelets have a weak thromboplastic ac tion in systems such as those described and that (2) weak thrombin and (3) serum fibrinolysin each have a significant potentiating action on this platelet effect Experiment 37 was even more satisfactory and controlled than the recent confirmatory Experiment 36

(7 B) EFFECTS OF ANTIPROTEASES ON VARIOUS 'THROMBO PLASTIC' ADDITIVES IN THROMBIN FORMING SYSTEMS

Preliminary Studies: These will be cited in later sections These clearly established the following facts needed for interpretation of the thrombin forming experiments

I None of the antiproteases had any inhibitory action on the thrombin fibrinogen reaction (see Experiment 43 p 117 and Table XLVI)

II In higher concentrations than those selected for the present ex periments trypsin was thrombinolytic and Loomis' fibrinolysin very feebly so requiring several days incubation in order to demonstrate this

III Again in too high enzyme concentrations to be significant in the present experiments a question of prothrombinolysis might arise 430 301 However Dr Lewis Ann C Howe and the author [299]

and supplied by Dr M Kunitz [323] (Rockefeller Institute Princeton N J) whose 'units' of activity are cited [353] Tryp. B a very satisfactory commercial trypsin (Fairchild Bros & Foster N Y) of which a stock 2% solution was made in 50% glycerol and borate buff (i e equal vols)

Anti proteases (Trypsin inhibitors) For antifibrinolysin see above P.I. pancreatic inhibitor (anti trypsin) highly purified (Lot P-A 97-1) Sharp & Dohme Labs courtesy of Dr L A Kazal (cf [353]) S.B.I. soybean inhibitor a crystalline preparation kindly supplied by Dr M Kunitz [272] (see above) N.B.I.: a re purified navybean inhibitor (see p 117) kindly supplied by Dr D E Bowman [59] (Indiana University)

All these antiproteases were prepared in a 0 1% solution in borate buffer The S B I was diluted to 0 01% before use Special testings of the antiproteases will be cited later (p 117)

Platelets (Plat.) cf p 54 Well washed platelets were obtained as follows 100 ml dog blood was collected into 13 ml of 3 4% trisod citrate Repeated centrifugings for a few minutes at 1000 r p m in the International Refrigerated Centrifuge were performed until red cells were no longer visible in the sediment The 'platelet rich' plasma was then centrifuged for 30 min at 5000 r p m and the platelet sediment resuspended and well washed at least three times by thorough mixing with successive 50 ml lots of 0 9% NaCl containing 1/8 vol 3 4% sod citrate (we later used Triton ' p 54) and a final washing in plain saline The final suspension concentrated in 1-2 ml saline was examined microscopically Wright stained smears revealed only granular platelet material with no leukocytes and only a rare erythrocyte

Experiment 37 (1949) Table XL

Purpose (as in Experiment 36) To study the thromboplastic action of platelets and its potentiation by (A) weak thrombin and (B) fibrinolysin

Method A two stage system similar to previous testings of thrombin formation

T.M.: 5 ml vol containing (with borate buff) Pro + AcG + Ca + plat with and without thr.* (p 111) or lysin

Clotting tests at 26°C 0 5 B F + 0 25 T M

Results These are shown in Table XL

Discussion This experiment is one which we have many times repeated to show *the much better thrombin formation in a system of* purified Pro + AcG + Ca + platelets when a tiny trace of thrombin is also added (test 2) It must remain an open question whether a test system completely devoid of thrombin (even minutest traces) might someday be obtainable and fail (?) to activate with platelets and Ca (in the presence of proconvertin AcG etc) Pending such a finding it seems to be established that platelets are an incomplete' thromboplastin which can be potentiated by thrombin The thr * used in Experiment 37 was a special preparation In fact it consisted merely of the

same prothrombin preparation in borate buffer which had been al lowed to stand with $CaCl_2$ for several weeks in the refrigerator (4°C) Tests showed that it was completely converted into thrombin since incubation with thromboplastin and AcG gave no further enhancement of its clotting potency Such a preparation seemed well chosen to avoid the introduction of any factors except the thrombin unless per chance there are some unknown factors in the (recalcified) prothrom bin itself With these unsupported reservations we must conclude that weak thrombin can act as a remarkable potentiator of platelet thromboplastin Since AcG is added this would not appear to be due to the known effect [492] of thrombin in converting proaccelerin to accelerin Attempts were made to run controls without platelets These showed only insignificant shortening of the clotting times in the parallel test (O) without activators (except for the Ca) This cited test (O) merely showed the trace of thrombin in the freshly prepared prothrombin solution and this could not be correlated with the weak thromboplastic action of the platelets shown in (1) The added thr * (p 111) (diluted) clotted a test fibrinogen in 750 sec when freshly prepared but rapidly lost potency on standing in glass

The fibrinolysin test (3) showed a potentiation of the platelet thromboplastic effect qualitatively resembling that of thrombin and even quantitatively similar in this particular experiment It is diffi cult to test a fibrinolysin for traces of thrombin because the enzyme lyses the test fibrinogen before it has a chance to clot with a very weak thrombin We did perform this control test however in the presence of antiproteases (esp antifibrinolysin) which seemed to rule out this possibility It is not possible therefore to deduce a common mode of action for these two dissimilar agents (thrombin and fibrinoly sin) with any factual knowledge currently available

Conclusion We present the experimental facts therefore which prima facie indicate that (1) platelets have a weak thromboplastic ac tion in systems such as those described and that (2) weak thrombin and (3) serum fibrinolysin each have a significant potentiating' action on this platelet effect Experiment 37 was even more satisfactory and controlled than the recent confirmatory Experiment 36

(7 B) EFFECTS OF ANTIPROTEASES ON VARIOUS 'THROMBO PLASTIC ADDITIVES IN THROMBIN FORMING SYSTEMS

Preliminary Studies These will be cited in later sections These clearly established the following facts needed for interpretation of the thrombin forming experiments

I None of the antiproteases had any inhibitory action on the thrombin fibrinogen reaction (see Experiment 43 p 117 and Table XLVI)

II In higher concentrations than those selected for the present ex periments trypsin was thrombinolytic and Loomis' fibrinolysin very feebly so requiring several days incubation in order to demonstrate this

III Again in too high enzyme concentrations to be significant in the present experiments a question of prothrombinolysis might arise 430 301 However Dr Lewis Ann C Howe and the author [299]

could not confirm this but found instead a marked susceptibility of AcG to the proteolytic actions of fibrinolytic enzymes

IV When thrombin formation is very slow and weak it is possible to encounter fibrinogenolysis by the enzymes before the weak thrombin has a chance to form a clot In fact this is indeed one of the author's [137] methods for assaying trypsin and other fibrinolytic proteases at high dilutions as will be illustrated in Experiments 44 and 45 pp *117 and 118 respectively*

V There are quantitative relationships between enzymes and the respective enzyme inhibitors which must be worked out and observed [291 292] Furthermore the enzyme inhibitors act in different ways [292] and some of them require a time period hence pre incubation with the enzyme in order to exert significant antiprotease effects This pre incubation is indicated by an asterisk (*) with designation of the anti enzyme used

All these considerations were carefully tested before using the previously *described* (pp 111 112) *reagents in the following groups* of tests

Experiment 38 (1948) Table XLI

Purpose To test antiproteases on platelet and trypsin additives in the thrombin forming system

Reagents Plat 0 1 ml conc suspension (p 112) Tryp : 0 01% Kunitz's crystalline trypsin (p 111) 0 3 ml P.I. 0 1% pancreatic inhibitor (p 112) 0 1 ml Afin 4% Loomis' anti fibrinolysin (p 111) 0 3 ml Pro 0 1% Seegers prothrombin (p 111) 0 2 ml AcG 0 1% Ware and Seegers' serum AcG (p 111) 0 2 ml Ca 0 5 ml 0 05M $CaCl_2$

Method and Results These are shown in Table XLI They demonstrate (1) weak thromboplastic action of platelets; (2) similarly weak thromboplastic action of trypsin alone; (3) marked potentiation of thromboplastic action when trypsin is added with platelets (4) inhibition of the last by P I and (5) similar inhibition by Afin*

Clot lysis This was followed over 3 weeks in these experiments It was noted in 3 days in (2) but was completely inhibited by the platelets (3) as well as by the antiproteases (4 5)

Experiment 39 (1948) Table XLII

Purpose To test antiproteases on platelet + fibrinolysin additives in the thrombin forming system

Reagents Same as in Experiment 38 except for (a) use of 0 4 ml 0 5% Loomis fibrinolysin (p 111) instead of trypsin and (b) additional test with 0 2 ml 0 01% S B I (soybean inhibitor p 112)

Method and Results *These are shown in Table XLII*

Discussion Test (1) of Experiment 38 (Table XLI) was also the control with platelets alone for the additional tests in Experiment 39

(Table XLII) (6) the fibrinolysin alone had a very weak thromboplastic effect as compared with the control (11) in which Ca and AcG alone caused no detectable thrombin in tests watched for ½ hr (7) the fibrinolysin however markedly potentiated the platelet thromboplastic action apparently like trypsin did in tests (3) of Table XLI (8) Unlike the trypsin experiment (4) however P I did not inhibit this action of fibrinolysin (9) Neither was Afln* able to cause any significant inhibition of the fibrinolysin's potentiating action again differing from the corresponding trypsin experiment (5) S B I (10) seemed to inhibit in the earlier phases although much less so later It will be shown (p 116) that this can be explained as due to a direct anti thromboplastic action of the S B I itself In fact it is somewhat significant that this was eventually largely overcome by the fibrinolysin preparation

Experiment 40 (1956) Table XLIII

Purpose To test antiproteases in thrombin forming systems in which the thromboplastic additives are (I) platelets only (II) platelets + antihemophilic globulin

Reagents Eluate dialyzed (p 81): to provide prothrombin proconvertin PTC etc (0 2 ml per 5 ml T M)

Plat 0 2 ml of a new preparation of human platelets (normal) p 54

AHG: Dr R H Wagner s antihemophilic globulin preparation described on p 77 (0 2 ml)

S.B.I.: 0 1 ml of a new 0 1% borate buffer solution of crystalline soybean inhibitor (Worthington Biochemical Lab Freehold N J)

P.I. Dr L A Kazal's pancreatic inhibitor preparation (p 112) 0 1 ml of 1% sol in borate buffer

N.B.I.*: Dr D E Bowman's re purified navybean inhibitor preparation (p 117) 0 1 ml of 0 1% sol in bor buffer

Afln*: Dr E C Loomis 1947 preparation (p 111) A 0 4% two day old extract (freed from insoluble material) in bor buff 0 3 ml The last two inhibitors* were pre incubated for 15 min at 28°C with the thromboplastic additives (I) or (II) respectively

T.M. s: Made up to 4 5 ml with borate buffer and then activated with 0 5 ml 0 04M $CaCl_2$

Results These are given in Table XLIII They reveal:

I (1) Platelets alone were significant activators

(2) S B I was markedly inhibitory (anti thromboplastic) especially in the earlier phases of activation

(3) P I and (4) N B I * were not inhibitory A very minor improvement was hardly significant

(5) Afln* was distinctly inhibitory but whether this was due to the very old preparation could not be determined as the last of this material was used up in this test

II (6) AHG caused the same important potentiation (? thromboplastin generation) of platelets as with cephalin (see test 7 Table XIII) Compare with test (1)

could not confirm this but found instead a marked susceptibility of AcG to the proteolytic actions of fibrinolytic enzymes

IV When thrombin formation is very slow and weak it is possible to encounter fibrinogenolysis by the enzymes before the weak thrombin has a chance to form a clot In fact this is indeed one of the author's [137] methods for assaying trypsin and other fibrinolytic proteases at high dilutions as will be illustrated in Experiments 44 and 45 pp 117 and 118 respectively

V There are quantitative relationships between enzymes and the respective enzyme inhibitors which must be worked out and observed [291 292] Furthermore the enzyme inhibitors act in different ways [292] and some of them require a time period hence pre incubation with the enzyme in order to exert significant antiprotease effects This pre incubation is indicated by an asterisk (*) with designation of the anti enzyme used

All these considerations were carefully tested before using the previously described (pp 111 112) reagents in the following groups of tests

Experiment 38 (1948) Table XLI

Purpose To test antiproteases on platelet and trypsin additives in the thrombin forming system

Reagents Plat 0 1 ml conc suspension (p 112) Tryp : 0 01% Kunitz's crystalline trypsin (p 111) 0 3 ml P.I. 0 1% pancreatic inhibitor (p 112) 0 1 ml Afln 4% Loomis' anti fibrinolysin (p 111) 0 3 ml Pro 0 1% Seegers' prothrombin (p 111) 0 2 ml AcG 0 1% Ware and Seegers' serum AcG (p 111) 0 2 ml Ca 0 5 ml 0 05M $CaCl_2$

Method and Results These are shown in Table XLI They demonstrate (1) weak thromboplastic action of platelets; (2) similarly weak thromboplastic action of trypsin alone (3) marked potentiation of thromboplastic action when trypsin is added with platelets; (4) inhibition of the last by P I ; and (5) similar inhibition by Afln*

Clot lysis This was followed over 3 weeks in these experiments It was noted in 3 days in (2) but was completely inhibited by the platelets (3) as well as by the antiproteases (4; 5)

Experiment 39 (1948) Table XLII

Purpose To test antiproteases on platelet + fibrinolysin additives in the thrombin forming system

Reagents Same as in Experiment 38 except for (a) use of 0 4 ml 0 5% Loomis fibrinolysin (p 111) instead of trypsin and (b) additional test with 0 2 ml 0 01% S B I (soybean inhibitor p 112)

Method and Results These are shown in Table XLII

Discussion Test (1) of Experiment 38 (Table XLI) was also the control with platelets alone for the additional tests in Experiment 39

future Qualitatively at least platelets seemed to be potentiated by trypsin in very much the same way as cephalin [149] (p 105) and weak thromboplastin [156] (pp 105 106) were We concluded that there was an important common denominator in these comparisons It could be a common thromboplastic phospholipid mobilized or made available to an enhanced degree by the disaggregating (Pope [379] cited [138]) action of the proteolytic enzyme Such an idea fits the author's ceph alin availability theory and could explain the significance of throm boplastic *enzymes' like trypsin* (cf p 104)

(7 C) CONTROL TESTING OF ENZYMES AND ANTIPROTEASES

Experiment 43 (1948) Table XLVI

Purpose To show that anti proteases have no significant effect on the thrombin fibrinogen reaction

Method Timing of the clotting of a test fibrinogen with pre incu bates of thrombin and the various antiprotease preparations 2 ml thrombin (bovine Parke Davis & Co s thrombin topical p 56 5 units/ml) + 2 ml antiprotease (or borate buff in control) incubated for periods of ¼ min to 1 hr and 0 5 ml samples of mixture tested on 0 5 ml B F Armour s (1%) p 55

Results These are given in Table XLVI They were negative with regard to any significant effects of (1) P I (p 112) 0 1% (2) S B I (p 112) 0 005% (3) N B I (below Experiment 44) 0 01% (4) Afin (p 111) 0 1% even with an hour s pre incubation with the thrombin

Experiment 44 (1956) Table XLVII(A)

Purpose To demonstrate proteolytic activity in a long preserved (>11 yr) trypsin preparation and to show its inhibition by two types of trypsin inhibitors

Method The fibrinogenolytic technique originally described by the author [137] in 1943

Reagents Borate buffer pH 7 7 (p 111) Thr bovine thrombin (Upjohn's p 56) 20 units/ml B.F. 1% bovine fibrinogen (Armour's $BaSO_4$ treated p 55)

Tryp The >11 yr old (commercial) trypsin preparation used in Experiment 35 (p 108) The 2% stock solution was diluted 100x with borate buff

N.P.I. a highly purified (reprecipitated with $(NH_4)_2SO_4$) antipro tease from navy beans prepared by Dr D E Bowman [59] and kindly supplied us in 1948 A fresh 0 1% solution of the dried preparation was made up in borate buff

P.I. A highly purified pancreatic trypsin inhibitor prepared Jan uary 2 1948 (Lot No P A 97 1) by Dr L A Fazal (Sharp and Dohme Labs Glenolden Pa) Fresh 0 1% solution in borate buff

L.M. (lysing mixture) 1 ml B F + 0 5 ml tryp + 0 5 ml inhibitor incubated at 28°C with testing of 0 2 ml samples at intervals on adding

(7) S B I was very antithromboplastic in this mixture also (cf (2))

(8) P I and (9) N B I * were non inhibitory

Experiment 41 (1948) Table XLIV

Purpose To test antiproteases in thrombin forming systems acti vated by Ca and tissue thromboplastin

Reagents and Method Same as in Experiments 38 and 39 ex cept for use of 0 5 ml tpln, A (p 90) as the only thromboplastic addi tive

Results These are given in Table XLIV They show:

(1) Marked activation with this strong tissue thromboplastin

(2) Its complete inhibition with S B I an important control find ing (cf Experiment 39)

(3) No inhibition by P I and (4) none by Afin *

Experiment 42 (1956) Table XLV

Purpose To test antiproteases in thrombin forming systems ac tivated by Ca and cephalin

Reagents and Method Same as Experiment 40 (I) namely us ing dog eluate (prothrombin etc) AcG borate buffer but substituting 0 1 ml 0 1% cephalin (p 71) for the platelets

Results These are given in Table XLV They reveal

(1) Satisfactory activation by the cephalin

(2) S B I (p 112 0 1 ml of 0 1% solution) was again anti thrombo plastic (cf tests (2) and (7) Table XLIII and test (2) Table XLIV)

(3) P I (p 112 0 1 ml of 0 1% solution) was not inhibitory neither was (4) N B I * (p 117 0 1 ml of 0 1% solution)

General Discussion of Experiments 38 42 Of the vari ous antiproteases tested the soybean inhibitor introduced complica tions because of its direct anti thromboplastic effects against plate lets (Experiment 40 (I)) or platelets + AHG (Experiment 40 (II)) tissue thromboplastin (Experiment 41) or cephalin (Experiment 42) The others were reliable inhibitors of the enzymes trypsin and fibrin olysin in the systems studied and had no significant antithromboplas tic effects of their own Antifibrinolysin in Experiment 40 (I) was a questionable exception Inhibition by antiproteases of the platelet thromboplastin potentiating action of trypsin was good evidence that this phenomenon depended upon the proteolytic actions of the pancre atic enzyme On the contrary the apparently similar potentiating (to platelet thromboplastin) effects of fibrinolysin were not removed by antiproteases in amounts sufficient to suppress all proteolytic action This unexpected difference could not be explained except by postulating some unknown factor in the enzyme preparations used A suggestion that it might be the 'Hageman factor' [401] was noted (p 26) in the introductory section but further exploration of this must await the

excellent degree of activity and was judged suitable for the tests with platelets in Experiment 36 The antifibrinolysin was also satisfactory Similar tests were made with the enzyme systems used in the 1947 49 experiments previously cited

(7 D) FIBRINOLYTIC PHENOMENA AND PLATELETS

The use of borate buffer with its mild bacteriostatic effect proved very valuable as a preservative in our earlier [156] prothrombin activation experiments and enzyme studies (ref [291]) In these sys tems it was often possible to demonstrate weak fibrinolytic enzymes requiring days or weeks to produce clot lysis We shall cite only those incidental observations which pointed to certain relationships of the platelets to proteolytic phenomena Chief among these was the demon stration of an antiprotease in platelets particularly noted in the inhi bition or retardation of fibrinolysis by trypsin This is evident in the clot lysis data of Table XLI (Experiment 38 p 114) References to platelet anti fibrinolysin' are given on p 51 We had observed it in Experiment 39 and other similar experiments in 1947 49

(7 E) THE 'AcG LIKE' FACTOR IN PLATELETS [476]

Experiment 46 (1950) Table XLVIII

Purpose To test for an accelerator factor in washed platelets using an AcG poor prothrombin activated by Ca and a strong tissue thromboplastin Comparison with varying concentrations of Ware & Seegers' AcG preparation

Method The usual two stage technique (1) without added AcG (2) with platelets and (3) and (4) with varying concentrations of added AcG

Reagents Pro One of Dr Seegers highly purified bovine pro thrombins Tpln, B; Soluplastin' (p 56) Plat p 112 AcG: p 111 T M.'s as described in Table XLVIII

Results These are given in Table XLVIII

Discussion The poor thrombin formation in (1) with tpln only indicated the accelerator deficiency in the chosen prothrombin In (2) platelets were found to have only a little effect Since there was already a strong thromboplastin present this small effect could have been due to some 'AcG like' factor in the platelet preparation Com parisons with the two additions of 'purified serum type AcG (3 4) sug gested that this platelet factor was roughly comparable to what amounted to a concentration of only 1:2 500 000 in the final thrombic mixture or 1 100 000 equivalent vols of platelets and AcG Questions as to the lability of 'platelet AcG' during the manipulations of platelet prepara tion were difficult to answer We have found significant amounts of platelet AcG in our more recent (1952 56) clinical routine tests dis cussed later (see Table LII)

to 0 2 ml Thr Successive prolongations of the clotting time indicate the rate and extent of the fibrinogenolysis

Note The P I acts immediately and is added just before the tryp sin The N B I * on the other hand resembles serum fibrinolysin in requiring a period of time to inhibit the proteolytic enzyme [292] Hence 20 min pre incubation of N B I with trypsin was allowed be fore adding the fibrinogen Borate buff was used to make up the 2 ml L M vol as needed

Incubates
1) *Control without enzymes*
2) Trypsin alone;
3) Tryp + N B I *
4) Tryp + P I

Results These are given in Table XLVII(A) They clearly show:
1) stability of fibrinogen in buffer (control)
2) marked fibrinogenolysis by the diluted old trypsin;
3) complete inhibition of lysis by the N B I *;
4) almost complete inhibition also by the P I

Conclusions This experiment affords evidence of the remarkable stability of trypsin at ordinary refrigerator temperatures (4°C) in glycerol borate buffer (equal vols) solution It indicates suitability of this old enzyme preparation for the special platelet potentiation tests of Experiment 35 (p 108) and anti heparin Experiment 47 (p 120) Similar tests were made on this preparation and on crystal line trypsin solutions preliminary to the 1947 1949 experiments (p 111 et seq)

Experiment 45 (1956) Table XLVII(B)

Purpose To test the proteolytic activity of an old (> 43 months) dog serum fibrinolysin preparation and its inhibition by antiproteases

Method The fibrinogenolytic technique [137] as used in Experi ment 44 also employing the same reagents except for: (a) replacing the trypsin by lysin a purified' fibrinolytic enzyme (plasmin or tryp tase) from dog serum prepared by Dr Jessica H Lewis and the au thor [291] October 29 1952 A 1:40 dilution in borate buffer had been preserved frozen at 20°C (b) Afin (0 4% in borate buff) was pre pared by 2 day extraction at 4°C of a November 1947 dried anti enzyme preparation of Dr E C Loomis (see p 111)

Data *These are shown in Table XLVII(B) They demonstrate:*
1) Stability of the control fibrinogen (buffer only)
2) Marked fibrinogenolysis by the old enzyme solution complete *in 10 min*
3) Inhibition of proteolysis when the enzyme was pre incubated for 15 min with 0 1% N B I * (equal 0 5 ml vols)
4) Similar inhibition by Afin * (also pre incubated 15 min with the lysin)

Conclusion This dilute enzyme preparation also preserved an

hence restore the platelet (+ enzyme) thromboplastic activity was very strikingly demonstrated by these tests

(5) The trypsin alone was only weakly thromboplastic This strongly suggested that the activity which was released from the heparin inhibition by trypsin was not that of the enzyme itself but must have come from the platelets

(6) The strong tissue thromboplastin (Soluplastin) was also able to overcome the inhibitory effects of heparin in this thrombin forming system This again confirms our 1939 Experiment (29 p 100 tests 5 6)

Discussion Unlike our earlier experiments (29 31) which used excessive amounts of heparin as commented upon on p 104 the present study (Experiment 47) employed concentrations of heparin which might be compared favorably with those in the therapeutic or physiological range Hence it is particularly convincing that such amounts of heparin were able so completely to inhibit the thrombo plastic action of platelets in our suitably chosen thrombin forming system Inability of this heparin however to antagonize tissue thromboplastin or trypsin + platelets was equally significant evi dence of the much greater potency of these thromboplastins

Conclusions The foregoing data not only suggest (1) that the usual anticoagulant effects of heparin are intimately associated with inhibition of the thromboplastic function of platelets but also (2) that platelets are much more akin to a simple phosphatide (? cephalin) as in Experiment 29 than to ordinary preparations of tissue thromboplastic extracts Quantitative interrelationships undoubtedly exist and an extremely weak tissue thromboplastin would closely resemble platelets and cephalin in these experiments However we do believe the facts line up behind the postulates of our 'cephalin availability theory (See p 143)

(7 G) ANALYTICAL FRACTIONATION OF PLATELETS

This line of investigation was not pursued in this thesis The reader may be referred to current work in this field by Dr W H Seegers of Wayne University Detroit; but the brief reference we have given to this on p 49 will have to suffice The one group of analytical data with which the present writer had some connection was that of Mrs Betty Nims Erickson and collaborators in the 1940 s cited on p 44 From these we wish to reiterate the impor tant finding (also credited to Chargaff [78] on p 44) that acetone insoluble phospholipid or cephalin is a major fraction of the platelet lipids

(7 H) QUESTION OF A 'FIBRINOPLASTIC FACTOR IN PLATELETS

The author s [134] term 'fibrinoplastic (see p 17) for factors which accelerate the thrombin fibrinogen reaction through some non specific colloidal or surface effect would seem to be appropri ate for such action of platelets (? fractions) as that claimed by Ware Fahey & Seegers [490] and allegedly confirmed by others (see p 51)

Conclusion These early experiments led us [476] (see p 50) to wonder whether the 'AcG like' factor described by Ware Fahey & Seegers [490] was a genuine platelet component or might merely be adsorbed onto the platelets from the plasma [222] Our tests which will not be cited in detail indicated the platelet accelerator to be of the plasma AcG type or proaccelerin (see p 26) More about platelet analyses for this factor will be given later in data on clinical cases (p 130)

(7 F) EFFECTS OF HEPARIN ON THROMBIN-FORMATION BY (I) PLATELETS; (II) PLATELETS + TRYPSIN; OR (III) TISSUE THROMBOPLASTIN

Experiment 47 (1956) Table XLIX

Purpose To study heparin inhibition of the thromboplastic action of (I) platelets and differences from (II) platelets + trypsin and (III) tissue thromboplastin

Method Essentially the usual method III two-stage technique of following thrombin formation with slight modifications of volumes to control for the heparin carried over into the thrombin fibrinogen (clotting test) mixtures

Reagents Eluate (dialyzed No 127) p 75 was used to provide prothrombin proconvertin etc 0 2 ml/5 ml T M

Plat Human platelets (p 54) 0 2 ml

Tryp : 0 5 ml of a fresh 1 100dil with imid buff sol (pH 7 3) of the 11 yr old stock (27) trypsin described on p 108

Tpln : 0 2 ml 'Soluplastin' (p 56)

Ca 0 04 M $CaCl_2$

Hep : Imid buff sal dilutions of heparin ('Liquaemin Organon Inc (Hoffman LaRoche) Orange N J) 0 5 ml of 2 units/ml hep in T M (b series): 0 1 ml of 0 2 units/ml hep in (c) series of fibr + T M tests

T.M.'s and clotting test mixtures (0 2 ml fibrinogen (dog) +0 1 ml (a) 0 2 units/ml hep or imid buff sol + 0 1 ml T M): are described in the caption of Table XLIX

Results (Table XLIX) show in:

(2) practically complete inhibition of platelet thromboplastic activity by heparin in concentration of 0 2 units/ml in the thrombin forming mixture

(1 a c) was the appropriate control supplying the same final concentration of heparin namely 2/4 of 0 2 = 0 1 unit/ml as in (2) tests

(1 a d) with no heparin were only a little better than (1 a c) thus showing that the effects of heparin in the concentrations tested were very minor on the thrombin fibrinogen reaction

(3) the significant trypsin potentiation of the thromboplastic action of platelets again confirms the data of Experiments 35 38 The only difference was that in Experiment 47 the final clotting tests were performed in the presence of heparin

(4) The ability of trypsin to overcome the heparin inhibition and

original (0) value The rapid drop in the platelet counts thereafter probably represents chiefly the removal of platelets by entanglement in the fibrin clot (B) The hemophilic blood started lotting in 25 min Averaging the 4 5 and 7 min tests the platelet count had fallen in this time to 76% only (137 3/182) of the original (0) value The pre clotting 10 15 min period i e 3 observations gave a similar value viz 87% within the 'experimental error Only after clotting was there an abrupt fall in the platelet count suggesting removal of the formed elements entangled in the fibrin clot

Conclusion There seems to be a significant preservation (80±4%) of platelets during the longer pre clotting period in hemophilic as compared with normal (32%) human blood This could reflect a dimin ished 'utilization' of platelets in the pre clotting phase of hemophilic blood Platelet counts after the onset of clotting are not significantly different and in all probability depend upon platelet removal by en tanglement in the fibrin clot

8 DARK FIELD MICROSCOPIC OBSERVATIONS OF PHOSPHOLIP IDS PLATELETS AND OTHER FORMED ELEMENTS OF THE BLOOD AND BONE MARROW

(8 A) FOREWORD

Siedentopf & Zsigmondy [441] applying the optical principles worked out by Abbe and Helmholtz first used the dark field method of microscopy It was quickly applied to the study of living cells and micro organisms by numerous investigators in Germany France and elsewhere The early history of its use for study of the blood was re viewed in 1913 by Aynaud and Jeantet [27] Aynaud [24 25 26] used it as well as ordinary miscroscopy in a monumental study of the al terations of the blood platelets in a work which deserves far more recognition than it has received by occasional brief mention in the more modern reviews [411 415] Stubel [459] in 1914 gave some good descriptions of the dark field appearances of platelets with ex cellent photomicrographs The present author demonstrated cellular and clotting appearances in a drop of human blood preserved at body temperature under the dark field microscope before the British Asso ciation for the Advancement of Science meeting at Cape Town in 1929 This initiated an interest pursued from time to time over many years leading to publications in South Africa [122] and in America [124 139] The following account summarizes those observations relating to the role of phospholipids in the alterations of platelets and other formed elements which occur after withdrawal from the body It is original more particularly in the novel explanation which is arrived at from an analysis of the data reviewed

(8 B) PLATELETS

1 Alteration during blood coagulation (Figure 18)

Method A simple small drop of finger prick blood is placed on a scrupulously clean slide and spread thinly with gentle pressure from

B L Travis and the present author in 1947-49 (p 115 of ref [476]) were unable to demonstrate any significant effect of (dog) platelets on the thrombin fibrinogen reaction

Experiment 48 (1956) Table L

Purpose To re study the effects of adding platelets to thrombin fibrinogen mixtures

Reagents Plat 0 1 ml of normal human platelet suspension (pp 54 112) Thr bovine thrombin (Upjohn's p 56) 0 1 ml of serial dilutions with imid buff saline (p 56) of 20 unit/ml stock solution Fibr 0 2 ml of (dog) $BaSO_4$ treated fibrinogen (p 55)

Method Clotting times (sec) at 26°C of thr + plat (or buff saline in controls) + fibr

Results are given in Table L They show no significant effects of the platelets (1) as compared with the controls (2)

Discussion The tests were meticulously performed The duplication of values in repetition of the last two measurements indicates the insignificant experimental error Essentially similar results were obtained with the 3½ yr old dog platelet preparation described on p 95

Conclusion We are unable to confirm any fibrinoplastic' action of platelets and wonder whether such findings by other workers may not be the result of some alterative (? denaturation) phenomena Denatured proteins often do have some effects on the thrombin-fibrinogen reaction [60]

(7 I) PLATELET UTILIZATION DURING CLOTTING

Experiment 49 (1955) Table LI

Purpose To investigate the rate of disappearance of platelets during the clotting of (A) normal and (B) hemophilic bloods

Method Bloods were collected by clean puncture of the antecubital vein using No 20 new needle and siliconized syringe (I) was immediately placed in a siliconized tube at 37°C and samples pipetted off at the stated intervals (Table LI) into a series of siliconized red cell hemocytometry pipettes and the platelet counts [62] made as soon as practicable thereafter (II) 4 5 ml of blood was mixed with 0 5 ml 0 1M sod citrate *in a siliconized pyrex bottle and held at 37°C until the* control period when its platelets were counted as usual

Results are given in Table LI

Data (A) The normal blood clotted soon after removal of the 6 min sample Averaging the 5 and 6 min tests i e just before clotting the platelet count had dropped in this time to 104 5/320 = 32½" of the

is far more common for them to remain for hours either anchored to the residue of the platelet body or occasionally breaking away and floating free The clubs and filaments are essentially similar to the vesicles except in shape and in the lack of particulate contents They are very delicate and oscillate vigorously with the jerkiness of typical Brownian movement They frequently become detached The feebly refractile double contoured free wriggling filaments are characteristic objects They often adhere to red cells and other objects We suggest as did Graszberger [472] for the erythrocyte filaments (see p 128) that a number of observers in the past have mistaken these for spirochaetal (or other) micro organisms and erroneously tried to explain certain diseases (e g Hodgkin's disease rheumatoid conditions etc) as 'infections' with such agents

From the body of the platelet we [124] have seen on one occasion during the earlier phases of alteration a single bright particle jerking in and out of the parent element in a manner resembling the agonal movements of the granules in dying leucocyte fragments as described by the author [122] in 1930 This could be regarded as evidence that the platelet is a necrobiotic cellular (see megakaryocyte) fragment Such outline of the body of the platelet as may remain visible after formation of the 'excrescences' exhibits delicate form changes for a short time but soon ceases to be distinguishable especially when several platelets adhere together The ultimate result of platelet alteration is the formation of an adherent granular matrix of large and small particles in which body outlines are no longer visible Adhering to the mass are numerous vesicles clubs and filaments and often several fibrin needles (see Figure 1 of [124])

2 Agglutination of platelet.

This characteristic phenomenon of normal platelet alteration was first described by Hayem [213 214] in 1877 1878 and especially studied in 1917 by J H Wright and G R Minot [,11] under the name 'viscous metamorphosis coined by Eberth & Schimmelbusch [113] in 1886

Under the dark field microscope it is seen that the platelets which happen to be thrown together while acquiring the stickiness previously noted during the period of swelling readily adhere to form a clump which is increased by the addition of other platelets that chance along It is this removal by agglutination and adherence to glass and other wettable surfaces rather than disintegration which makes it so difficult to find platelets in serum from clotted blood and also renders any attempt at counting of the platelets invalid if even a trace of clotting has occurred Aynaud [24] and others including ourselves [124] used paraffined surfaces to minimize these clumping phenomena The more recent introduction of non wettable siliconed surfaces is an improvement over these earlier methods which we have frequently used in more recent years and which has been commented on in some detail by Marjorie B Zucker [518] as noted on p 38 The phenomena of platelet clumping and partial disintegration (or 'alteration) normally in vitro make it difficult to study specific platelet agglutination and platelet lysis e g after sensitization to certain drugs or antiplatelet sera [1] Such studies must be performed with extremely careful technique and the use of siliconed surfaces

a No 0 coverslip It is examined under the oil immersion lens of a good dark field microscope with an adjustable diaphragm in the objective to permit best optical resolution of the minute details observed A bright tungsten band or similar evenly focussed (e g Burton lamp) illuminant is adjusted for optimal results and the cardioid condenser (dark field) is accurately centered The heat of the lamp usually warms up the blood drop sufficiently without requiring the 37°C chamber (enclosing the whole microscope) which was used in our earlier observations

Observations There are no significant alterations in the chylomicrons ('hemoconia') erythrocytes and leucocytes [124] in the few minutes which elapse before the appearance of the fibrin 'needles' which indicate the clotting of the blood The platelets, however typically 'alter' so rapidly that it is rare to observe them in their normal circulating form namely that of a tiny ovoid disc about ¼ ⅓ the diameter of an erythrocyte and with a delicate feebly refractile contour a clear translucent periphery (optisch leer Stübel [459]) and a few central granules of varying refractility which are devoid of Brownian or other movement There is no trace of any nucleus Viewed in profile the platelet appears 'kennetjie' ('tip cat' or 'batonnet' Aynaud [24]) shaped or fusiform ('spindelformig' Stubel [459] see p 35 Altered forms of platelets occur in a matter of seconds after withdrawal of the blood First there is a swelling into a spherule the shape of which is quite evident when it happens to be rolling over in fluid currents in the plasma This form has an even fainter outline but more distinct inner granulation consisting of the larger and brighter particles formerly observed together with a background ('granulomere') of finer hazy granules only faintly visible It attains to three or four times the size of the original platelet and evinces a 'stickiness' which causes it to adhere to the slide or coverslip Once adherent its outline may continue to expand apparently from several foci until a diameter of some 10µ is reached We term this a spreader form It is seen in only a small minority of the altering platelets however Much of the commonest change is that which has been frequently described by earlier observers namely the stellate form (see Plate 1 of Figure 18) This shows a number of processes or as we prefer to call them 'excrescenses' protruded from the margin of the platelet and taking up practically all the material of the outer layer or 'hyalomere' The excrescences are of three kinds viz (1) rounded or 'vesicular' ('bosselures' Aynaud 'kugelige Bläschen' Stübel) which are most frequent under these simple conditions (see Plate 3 of Figure 18) (2) club like and (3) filamentous (see Figure 3 in [124])

The delicate vesicles of (1) above seem to hover like a captive balloon at the surface of the platelet After a certain degree of swelling they have been observed to shrink temporarily with an adjacent eddying of hemoconia (chylomicrons) suggesting release of contents into the surrounding plasma As they gradually swell again there appear a few granules some apparently entering in from the body of the platelet *and these granules show vigorous Brownian movement* suggesting that the contents of the vesicle have now become liquified Several vesicles usually form from each platelet and their size varies up to 5 or 6µ Sometimes they rupture completely and disappear but it

is far more common for them to remain for hours either anchored to the residue of the platelet body or occasionally breaking away and floating free The clubs and filaments are essentially similar to the vesicles except in shape and in the lack of particulate contents They are very delicate and oscillate vigorously with the jerkiness of typical Brownian movement They frequently become detached The feebly refractile double contoured free wriggling filaments are characteristic objects They often adhere to red cells and other objects We suggest as did Graszberger [472] for the erythrocyte filaments (see p 128) that a number of observers in the past have mistaken these for spirochaetal (or other) micro organisms and erroneously tried to explain certain diseases (e g Hodgkin s disease rheumatoid conditions etc) as 'infections' with such agents

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3 Platelets and fibrin

The often reported finding of fibrin needles or filaments adhering to altering platelets is in fact less important than the observations: (1) platelet alterations commence long before fibrin appears (2) many fibrin needles can be seen under the dark field to appear de novo out of the background of the clear plasma by no means requiring the platelets (or other visible object) as foci for their formation

4 Platelet preservation

The mere use of a non wettable surface e g silicone sans anticoagulant has only a minor effect in delaying the typical platelet alterations which soon show all the appearances which we have described Of course it is highly probable that complete maintenance of non wettability is impossible under the conditions of observation and that there is bound to be some air liquid interface

Anticoagulants afford a much more practical means of controlling platelet alterations Aynaud's studies see p 123 must be given full credit for the thorough investigation of very many added agents investigated for their effects on platelet alterations We have merely used citrate oxalate and more recently sequestrene-Na_2 (EDTA see p 79) to confirm the ability of the decalcifying anticoagulants to preserve platelets By preservation we mean that the alterations which do still occur are much less disintegrative All changes are greatly retarded and there are some definite differences from the sequence of events which we have described above Agglutination of the platelets is particularly delayed and may be negligible even after days especially with sequestrene combined with the use of siliconed surfaces

When citrated platelet rich plasma is placed on an ordinary glass slide for dark field observation many single platelets are observed floating in the plasma Some however sediment and adhere to an extent sufficient to resist the small fluid currents always seen in these wet preparations Both free floating and adherent platelets are rather more refractile than in the simple blood drop The tiny disc form frequently well seen edgewise preponderates at first and may be regarded as the normal form Its outline is well defined and there is a clear hyalomere and a few central stationary granules (granulomere) It soon begins to show delicate short wavy filamentous excrescences from one or more points along the edge Confirming Stübel [459] we have sometimes seen these filaments form withdraw and reform but they soon remain extended and irreversible After a few minutes they appear to become more definitely rigid They seem to extend further over the course of several hours and may reduce the body of the platelets to insignificance Sometimes a single very long filament extends from the small granular remnant of the body in what we have termed a sperm like form [124] A number of rounded excrescences usually one (or two) to a platelet may also be observed Unlike the vesicles of unpreserved platelets however these can be seen edgewise to be disc like Hence we [124] use the term plaquette form Except with vigorous artificial manipulation it is unusual for either the filiform or plate-like excrescences to become detached Preserved platelets may be washed with saline (0 85% NaCl) in silicone and kept in the frozen stage (20°C)

Recalcification results in a re acceleration of the retarded alterative phenomena in the platelets and if the plasma is still present fibrin formation occurs a few minutes later The recalcified platelets swell adhere clump and (partially) disintegrate in much the same manner as in unpreserved blood and the phenomena are essentially similar in recent preparations and in long preserved platelets (in silicone at 4°C) recalcified as usual See Plate 2 of Figure 18 It is rather remarkable to see the rigid excrescences soften and swell and vesiculate often involving the whole of the platelet except for most of the granulomere Granules evincing Brownian movement may appear in the vesiculated forms

Effects of varying citrate concentrations were minutely studied in our 1934 publication [124] to which reference may be made for details The important conclusion was that the concentrations of decalcifying agent which prevented clotting also 'preserved the platelets to the extent of maintaining rigidity of excrescences lessened clumping and absence of 'fluidity' of platelet content We regard this as significant evidence that calcium has a specific role in the special (liquefactive') disintegration phenomena by which the platelets come to play a very important role in the blood clotting phenomena Osmotic imbibition of water by platelets (1) in hypotonic solution e g on diluting with distilled water or (2) on adding saponin (see Plate 4 of Figure 18) in the presence of citrate do not result in plasma clotting

The conclusion we [124] reached in 1934 was that the platelet 'alterations consist fundamentally of a 'myelin figure' formation from their surface phospholipids (see later) and that calcium by its influence on the phase relations of the lipoid water system specifically directs the water into the fatty platelet material thereby swelling and disrupting it This appears to be a significant contribution ordinarily to the platelet release of factors which the platelets contribute to the blood clotting system

(8 C) MEGAKARYOCYTES [139]

Bone marrow freshly obtained from the femur or tibia of anesthetized rabbits was quickly mounted in autologous serum previously obtained from heart puncture blood of the same animal Dark field microscopic appearances are shown in Plates 5 and 6 of Figure 19

In the former no anticoagulant was used and vesicular excrescences containing granules oscillating with Brownian movement are seen at the periphery of the megakaryocyte (in a small proportion of the giant cells observed) The excrescences are very similar to ordinary platelet alterations and like the latter often become detached In the second series of observations the marrow preparation was citrated (Plate 6) This suppresses the slow amoeboid movement of the megakaryocytes and causes them to show a well defined outline and a fixed glassy appearance of their very granular cytoplasm (very similar to leukocyte appearances in citrated blood preparations) A very few vesicles (see Plate 6) continue to appear however but are smaller more refractile devoid (or almost) of granules and resemble the plaquette form of correspondingly treated platelets (p 126)

We have never observed a living megakaryocyte fragmenting into platelets [510] and believe the phenomena described are absent or less

marked in the less mature megakaryocytes They were not observed in polykaryocytes (osteoblasts) which are unrelated to platelet origin

(8 D) THROMBOCYTES [139]

The nucleated thrombocytes of amphibia (frog) reptiles (turtle) fish (fresh water sun fish) and birds (fowl) have been studied under similar conditions Plate 7 (Figure 19) shows an altered frog thrombocyte lying on top of a large fibrin filament Note the vesicular bodies derived from the thrombocyte some still attached and others released for some distance As frequently noted in the earlier literature on thrombocytes their disintegration is often very sudden and explosive in character This scatters excrescences chiefly vesicles and granular material to some distance from the parent thrombocyte as well shown in Plate 8 (Figure 19) in the case of the sun fish observation It is noteworthy however that many vesicles persist in the region of the original adherence and disintegration of the thrombocyte The dark field observations confirm the essential similarity of the alterations of thrombocytes megakaryocyte margins and mammalian platelets

(8 E) ERYTHROCYTE STROMATOLYSIS ETC

Furchgott [182] in 1940 made dark field observations of altering erythrocyte ghosts (stroma of hemolyzed red cells) in contact with lyotropic salt solutions such as lithium perchlorate or potassium thiocyanate He was apparently unaware of our earlier data on platelet alterations and independently suggested the 'myelin figure' explanation of the phenomena observed His search of the red cell literature revealed several earlier observations (e g Oliver [355] Kite [267] Auer [23] and others) of essentially similar appearances from both hemolyzed and non hemolyzed red calls In some observations made by the present author in Cape Town in 1930 and recorded in the photomicrograph reproduced in Plate 9 Figure 20 'stromatolytic' filaments were observed extending from the margins of young erythroblasts in a drop of heart blood from a rabbit embryo Plate 11 Figure 20 photographed by an erstwhile graduate student (P H Ralph) working under the author s supervision at the University of Michigan in the early 1940's shows apparently similar thread like processes connecting points at which normal adult human red cells have apparently touched and then withdrawn from one another Plate 10 Figure 20 also prepared by Dr P H Ralph is a striking example of the spiculated stromatolytic appearances occurring in vitro in slide (dark field microscopy) observations of a nucleated red cell from a tadpole [139]

Conclusion. There is clearly a common phenomenon connected with *surface alterations of the several varieties* of blood and bone marrow formed elements collectively considered in the foregoing observations The basis for attributing these phenomena to a myelin figure formation from the surface phospholipids of these formed elements follows from the next series of observations

(8 F) MYELIN FIGURE FORMATIONS FROM PHOSPHOLIPIDS

R Virchow [483] first described myelin forms (figur s as micro scopical appearances at the edges of nervous tissue observed in con tact with watery solutions He interpreted them in terms of surface (interphase) relations between the lipoid and aqueous phases J B Leathes [284] studied them very extensively in 1925 He photographed at successive ½ minute intervals the large myelin figures appearing at the edge of a film of lecithin in contact with a wide variety of watery solutions chosen to illustrate the effects of (a) specific ions (b) osmotic concentration and (c) adsorbed substances The myelin forms were readily produced by distilled water Leathes noted a suppressive or inhibitory effect of calcium ions said to be overcome by 0 01 M NaOH and by cholesterol Other retarding and many favoring agents were recorded by this observer The present author in 1934 [124] repro duced some of the lecithin experiments of Leathes Plates 12A and 12B (Figure 20) show the dark field appearance (400x magnif) of the edge of the phospholipid film: (A) at the start in contact with air showing only the linear highly refractile margin and (B) 3 minutes after contact with distilled water showing the typical large (initial) myelin forms flowing out into the watery phase (upper right part of photo) Clearly visible in the original but poorly reproduced in the photomicrograph (and therefore retouched) is the translucent border as described by Leathes [284]

Our observations established one or two new points which we be lieve to be of considerable significance

Firstly all watery solutions cause an initial myelin figure outflow (i e into the aqueous phase) which is followed by a further expansion after a 5 10 minute 'delay period '

Secondly the dark field method permits interpretation of Leathes translucent border as a reciprocal phenomenon namely a penetration of the water into the lipid phase The secondary expansion phase we observed to be very dependent upon the particular ions in the watery solution If distilled water or sodium chloride (and similar salt) solu tions were used the secondary myelin figure outflow went on with increasing speed and vigor but the inflow of watery phase into the lipid was limited and showed little further penetration beyond that seen in the initial phase With calcium salt solutions these phase relations were reversed There was the same initial myelin figure outflow but little extension after the delay period Secondarily however the in flow of this solution into the lipid proceeded apace and without limit extending the 'translucent border throughout the lipid portion Our important conclusion was that calcium is specific in directing water into the lipid phase as is well known from the work of physical chem ists with phase relations of calcium and sodium salts in soaps and other fat water emulsions

To Leathes conclusion that myelin forms are due to surface growth and localized imbibition of water we would add that calcium ions specifically g neralize the imbibition and cause a 'reverse mye lin figure appearance as can be seen very clearly in dark field obser vations of lecithin films

Applying these ideas to platelet (etc) alterations we conclude that the calcium has the specific role not of causing the excrescence formations (which can occur in citrate etc) but in the liquefaction of the excrescences By directing water into the altering platelets or thrombocytes calcium plays an important role in their peculiar disintegrative phenomena What about the nature of the lipid itself? In our 1945 observations [139] we were able to include some dark field observations on other phosphatides namely the purified preparations of Dr J Folch (of the Rockefeller Institute New York) which are described on p 98

The chief additional information obtained was that cephalin of the several phospholipids studied yielded myelin forms which in size and appearance e g vesicles were often so similar to the above described platelet megakarocyte and thrombocyte alterations that it seemed reasonable to regard them as identical

Actually the fundamental myelin figure phenomenon was common to most of these phosphatides in contact with watery solutions and calcium salts caused a granular deposition penetrating into the lipid and interfering with its hydrotropic dispersion into the watery phase The strength of the salt solutions had only minor effects suggestive of some membrane phenomenon in which water penetration rates are partly controlled by osmotic considerations

The inositol phosphatide (III) behaved peculiarly in that it went directly into colloidal suspension as chylomicra' instead of showing 'myelin forms ' This is evidently representative of the behavior of the bulk of lipoidal and lipoprotein materials of plasma

Concerning the protein components of the surfaces of platelets etc our experiments have nothing to say

(B G) CONCLUSION

In summary then our comparisons of the dark field microscopical appearances of (a) water phospholipid films and (b) in vitro 'alterations' of platelets megakaryocytes thrombocytes and certain erythrocytes indicates the fundamental importance of myelin forms (figures)' and related phenomena at water lipid interfaces We believe these to be extremely primitive and essentially non vital phenomena of lipid rich surface membranes Not only is there an hydrotropism of the lipid material causing the appearance of surface 'excrescences' of the various types described but there is also a specific role for the calcium ion This reverses the phase relationships and directs water into the fatty phase thus contributing to the peculiar disintegrative phenomena studied Cholesterol proteins osmotic factors pH and perhaps other factors need additional study as further possible modifiers of the basic phenomena which we have described

C CLINICAL CASES WITH PLATELET PROBLEMS

FOREWORD

Our research problem 1950 55 with regard to the investigation of clinical cases with hemorrhagic and related disorders was largely planned and executed by Dr Jessica H Lewis Research Associate

and Co Investigator with advice assistance and some participation by the author Invaluable technical assistance was rendered by Francis M Morgan Doris C Ferguson Iris Rudin Ann C Howe and Bertha G Jackson Since 1953 a graduate student Mr James W Fresh has made many able contributions especially to Group I studies (see below) and in the past year Miss Mary Jane Patch has been a most helpful collaborator The staffs of the University s (North Carolina) Memorial Hospital Duke Hospital the Veterans' Hospital and ORINS Hospital have cooperated with the patient material as have a number of private practitioners We are indebted to Dr Marjorie B Zucker of the N Y U College of Dentistry Physiology Department for the serum serotonin assays [519] used to assess the platelet vasoconstrictor factor (Item 17 see Tables)

In preparing the data for this thesis the author has proceeded independently and has not consulted Dr Lewis who now has her own Research Laboratory at the University of Pittsburgh Gratefully acknowledging the leading role of Dr Lewis and the individual contributions of each member of our group the author nevertheless assumes sole personal responsibility for the following presentation and analysis of the data selected

CASES represent some 86 patients and 32 normal mothers and their infants They are classified into nine groups No attempt will be made to analyze them from the clinical viewpoint Rather they represent merely a physiological contribution of analytical laboratory methods with which to approach a rational diagnosis and understanding of the clinical hemostatic problems Our groups' are chosen from this physiological viewpoint to serve the purpose of this thesis inquiry

Methods for the most part have been described on pp 53 62 and will not be further elaborated An itemization (1 30) facilitates organization and reference In the first Table (LII) the mean and range of test values are given for (A) normal healthy adults (randomized as to sex and age) (B) parturient mothers and (C) their normal newborn infants (cord bloods)

0 ± (i e trace or minor) + ++++ indicate relative degrees of (7) bleeding tendency and (12) clot retraction (in glass) Retraction was also observed in silicone (cf 11) but this will not be reported since the differences from (12) were relatively minor In group summaries the number of cases was divided for each test into the following categories:

- Pos significant alteration of the test value
- N g not significantly different from the normal (norm)
- ? doubtfully significant
- N.T.: no test performed

Results are presented in Tables LII LX

GROUP I (A) Normal Adults (B) Parturient Mothers (C) Normal Newborn Infants (Cord Bloods) ref [172] Table LII

(A) Normals were from healthy adults randomized as to sex and age They were mostly medical students staff and hospital personnel including a few colored helpers The means of the various tests were

Applying these ideas to platelet (etc) alterations we conclude that the calcium has the specific role not of causing the excrescence formations (which can occur in citrate etc) but in the liquefaction of the excrescences By directing water into the altering platelets or thrombocytes calcium plays an important role in their peculiar disintegrative phenomena What about the nature of the lipid itself? In our 1945 observations [139] we were able to include some dark field observations on other phosphatides namely the purified preparations of Dr J Folch (of the Rockefeller Institute New York) which are described on p 98

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GROUP III: Hereditary Thrombocytopathic Purpuras Case 3: Glanz mann s Thrombasthenia Cases 4 5 Pseudohemophilia with Platelet Problems Table LIII B

Case 3 (N P) was a severe bleeder (7) with Pos Fam Hist (6) The plat count (230 000) was normal but clot retr (12) was absent and the bleeding time (9) and T T (8) were very significantly Pos Plat accel (16) was not tested and plasma proaccelerin (29) was a low normal (62%) Plat tpln (15) and ser serotonin (17) were normal (100%) Test 19 was Neg but serum antithr (20) seemed to be in creased Prothr consumption (25) was normal (95%)

Conclusion This rare case seems to be one of the very few re ported (ref [383]) which conform to Glanzmann s [188] 'hereditary hemorrhagic thrombasthenia '

Cases 4, 5 (A C and S C mother and daughter) presented a famil ial (6) disorder which corresponded most nearly to the classical de scriptions [484 485 486 260 262] of pseudohemophilia (ref [383 453]) Case 4 was a 52 year old white woman giving a +(+) history of bleed ing tendency (7) since 19 years of age The chief manifestations were ecchymoses and a moderately severe post operative hemorrhage Bleedng time (9) and clotting times (10 11) were normal as was clot retr (12) Her plat count (14) was normal (238 000) but the plat tpln (15) significantly reduced (50%) and plat accel (16) a low normal (50%) Serot (17) N T The T T (8) was significantly +++ Prothr consump tion (25) was normal (>95%) as were AHF (22) PTC(23) and other tests Plasma proaccel (29) and proconv (28) N T Fibr (21) was elevated

Case 5 the daughter 20 years old had also suffered +(+) ecchy motic bleedings Her plat count (14) was a low normal (104 000) and the plat tpln (15) 25% and plat accel (16) 20% unequivocally re duced Serot (17) N T Plasma proaccel (29) was normal (90%) AHF (22) likewise and probably PTC (23) 60% Prothr consumption (25) was quite normal as were other tests including a slightly ques tionable (71%) (recip see p 57) prothr time (26) Clot retr (12) was a satisfactory +++ Fibr (21) was elevated

Discussion In these two cases of pseudohemophilia the platelet problems were relatively minor and functional (thrombocytopathic) They must be regarded as of questionable clinical significance in a type of hereditary disorder which appears to be primarily a vascular dysfunction We had six other cases of pseudohemophilia two of hered itary telangiectasia and two with bleeding hemangiomata none of whom had any platelet problems and whose tests therefore are irrelevant to the present inquiry

Acquired deficiencies of platelet functions will be presented in the other groups The uremia cases (Group VIII) are especially interest ing (p 136)

GROUP IV: Acquired Thrombocytopenia (Cases 6 30) Table LIV

These 25 cases were clinically diagnosed as follows:

statistically determined on significantly large groups usually more than 50 cases

(B) Mothers The 32 cases studied permitted venepuncture during parturition They all appeared healthy and well and gave no history of recent infections antibiotic or excessive salicylate therapy dietary inadequacy or gastrointestinal disturbance The obstetrical anesthesia was non contributory Pregnancy labor and the puerperium were uncomplicated by toxemia hemorrhage or thromboses

(C) Infants The 32 newborn babies yielded blood samples from the umbilical cord before it had stopped pulsating All babies were delivered without trouble were uninjured and appeared normal None developed any bleeding tendency Some mothers and some babies received vitamin K questions concerning which are answered in the cited references [172 173]

Test Results See Table LII For purposes of this thesis comment will be limited to the noting of the absence of any platelet problems in (B) and (C) All tests in this regard fall within normal (A) limits A single exception (10%) of low platelet accelerator (16) in (C) was of doubtful significance (possible lability)

The many interesting findings in other tests are fully dealt with in the cited publications and include data on another 125 newborns and most of their mothers which are not included in the present more completely studied group

GROUP II Congenital (Familial) Thrombocytopenia (Cases 1 2) Table LIII A

Because of their unusual interest (ref [453]) these cases will be reported individually in Table LIII A

Case 1 (A B) was examined on two occasions (a) February 1 1954 before treatment and (b) April 20 1955 a few days after splenectomy In tests (a) the plat count (14) was very low (38 000) and assays for specific platelet factors (15 18) were not attempted Clot retr (12) was almost absent but prothr consumption (25) was amazingly normal The child had bleeding symptoms (7) at this time dating back to shortly after birth Bleeding time (9) was very prolonged and the T T (tourniquet test) (8) was strongly Pos

Family histories (F H) (6) will not be detailed in the present studies but were significantly Pos in Cases 1 and 2

In tests (b) after splenectomy all test values were restored to normal with the questionable exception of a 50% plat tpln (15) The patient developed no further bleeding problems in a 12 month followup

Case 2 (D D) was not a bleeder (7) despite the Pos Fam Hist (6) Nevertheless his plat count (14) was low (98 000) and tests 8 12 15 and 16 were significantly abnormal He should therefore be regarded as a potential bleeder

Discussion Case 31 (R G) was a 63 year old colored man with primary adenocarcinoma of the stomach and generalized metastases At autopsy the bone marrow was found to be hyperplastic with a greatly increased number of megakaryocytes He was examined four or five times between June 23 1953 and his demise on August 27 1954 The cited tests were on the first occasion but the thrombocytosi persisted at similar levels through June 29 1954 He developed a large hematoma in the upper right arm inexplicably Except for a low plat accel (16) on January 6 1954 all tests of platelet function including clot retr (12) and prothr consumption (25) were normal Serotonin (17) was not tested however The fibrinogen (21) was a borderline (200 mg) on January 6 1954 and plasma proaccel (29) low (20%) on the same occasion These findings could go with a fibrinolytic enzyme activation which might have been overlooked in recording of a glass c t (10) of 1 min and ++++ clot retr (12) on that occasion We do not feel sure of the crude fibrinolysin test however unless all the clot dissolves within 24 hours

Case 32 (J L H) was a 64 year old white male long known to suffer from polycythemia rubra vera Gout was an interesting complication (ref [503]) He had received P^{32} (red cell destructive) therapy at the Veterans' Hospital and we examined him twice (March 30 1954 and April 8 1955) after such courses of treatment when he was showing some bleeding symptoms (cf Group IX) Despite the extremely high platelet counts (14) the platelet function tests (15 16) and esp (17) serotonin were significantly depressed So were the prothrombin and related factors (26 29) but not the prothr consumption (25) although this might be misleadingly calculated because of the lowered level of the original plasma prothrombin

Case 33: (E M) was a 23 year old white male leukemic whom we have excluded from Group VI solely because of the unusual thrombocytosis Serotonin (17) was not tested but the other platelet function tests were normal There was some hypoprothrombinemia (27) and prolonged prothr time (26) which might have to do with liver function This case showed no hemorrhagic symptoms

GROUP VI Leukemias with Thrombocytopenia or Thrombocytopathia Problems (Cases 34 67) Tables LVI A LVI B

Besides the thrombocytosis case (No 33 Group V) platelet problems of one kind or another presented in 34 more (i e all but 4) out of 39 cases of leukemias studied The remainder of these test data will be separated into two groups (A B) by arbitrarily dividing them at a 100 000/mm level of the platelet count

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(A) 11 acute ITP (idiopathic thrombocytopenic purpura) of which 3 may have been due to drugs and 3 to infections namely (1) respiratory infection in a 17 mo old child (2) diphtheria in a 4½ yr old child and (3) infectious (? toxic) hepatitis in an 18 yr old white girl
(B) 7 chronic ITP including one of two months duration associated with rheumatoid arthritis in a 15 year old girl
(C) 5 were associated with anemias namely (1) two aplastic anemias (2) two macrocytic anemias one pernicious and the other occurring during pregnancy in a 22 year old woman and (3) one with Banti's syndrome
(D) 2 were associated with metastatic (bone) carcinomata in elderly males with primary prostatic malignancy

Test results are given in Table LIV in summary

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A small incidence (see Table) of other thrombin factor deficiencies is unexplained It is possible that liver function is occasionally disturbed perhaps by hemorrhage The elevated fibr (21) in 9 cases may be evidence of a liver reaction The low plasma proaccelerin (29) in 3 cases does not reflect the many more 19 cases low values for plat accel (16) Plasma thr c t (19) was definitely prolonged in 15 cases and slightly so in 8 more cases We do not yet know the possible significance of this finding

GROUP V Thrombocytosis (Cases 31 33) Table LV

These three cases present different clinical disorders but show in common very elevated platelet counts (14) Two 31 and 32 had bleeding symptoms

Results are detailed in Table LV

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marked in the less mature megakaryocytes They were not observed in polykaryocytes (osteoblasts) which are unrelated to platelet origin

(8 D) THROMBOCYTES [139]

The nucleated thrombocytes of amphibia (frog) reptiles (turtle) fish (fresh water sun fish) and birds (fowl) have been studied under similar conditions Plate 7 (Figure 19) shows an altered frog thrombocyte lying on top of a large fibrin filament Note the vesicular bodies derived from the thrombocyte some still attached and others released for some distance As frequently noted in the earlier literature on thrombocytes their disintegration is often very sudden and explosive in character This scatters excrescences chiefly vesicles and granular material to some distance from the parent thrombocyte as well shown in Plate 8 (Figure 19) in the case of the sun fish observation It is noteworthy however that many vesicles persist in the region of the original adherence and disintegration of the thrombocyte The dark field observations confirm the essential similarity of the alterations of thrombocytes megakaryocyte margins and mammalian platelets

(8 E) ERYTHROCYTE STROMATOLYSIS ETC

Furchgott [182] in 1940 made dark field observations of altering erythrocyte ghosts' (stroma of hemolyzed red cells) in contact with lyotropic salt solutions such as lithium perchlorate or potassium thiocyanate He was apparently unaware of our earlier data on platelet alterations and independently suggested the myelin figure explanation of the phenomena observed His search of the red cell literature revealed several earlier observations (e g Oliver [355] Kite [267] Auer [23] and others) of essentially similar appearances from both hemolyzed and non hemolyzed red cells In some observations made by the present author in Cape Town in 1930 and recorded in the photomicrograph reproduced in Plate 9 Figure 20 'stromatolytic filaments were observed extending from the margins of young erythroblasts in a drop of heart blood from a rabbit embryo Plate 11 Figure 20 photographed by an erstwhile graduate student (P H Ralph) working under the author s supervision at the University of Michigan in the early 1940's shows apparently similar thread like processes connecting points at which normal adult human red cells have apparently touched and then withdrawn from one another Plate 10 Figure 20 also prepared by Dr P H Ralph is a striking example of the spiculated stromatolytic appearances occurring in vitro in slide (dark field microscopy) observations of a nucleated red cell from a tadpole [139]

Conclusion. There is clearly a common phenomenon connected with surface alterations of the several varieties of blood and bone marrow formed elements collectively considered in the foregoing observations The basis for attributing these phenomena to a myelin figure formation from the surface phospholipids of these formed elements follows from the next series of observations

(8 F) MYELIN FIGURE FORMATIONS FROM PHOSPHOLIPIDS

R Virchow [483] first described myelin forms (figures as micro scopical appearances at the edges of nervous tissue observed in con tact with watery solutions He interpreted them in terms of surface (interphase) relations between the lipoid and aqueous phases J B Leathes [284] studied them very extensively in 1925 He photographed at successive ½ minute intervals the large myelin figures appearing at the edge of a film of lecithin in contact with a wide variety of watery solutions chosen to illustrate the effects of (a) specific ions (b) osmotic concentration and (c) adsorbed substances The myelin forms were readily produced by distilled water Leathes noted a suppressive or inhibitory effect of calcium ions said to be overcome by 0 01 M NaOH and by cholesterol Other retarding and many favoring agents were recorded by this observer The present author in 1934 [124] repro duced some of the lecithin experiments of Leathes Plates 12A and 12B (Figure 20) show the dark field appearance (400x magnif) of the edge of the phospholipid film (A) at the start in contact with air showing only the linear highly refractile margin and (B) 3 minutes after contact with distilled water showing the typical large (initial) myelin forms flowing out into the watery phase (upper right part of photo) *Clearly visible in the original but poorly reproduced in the* photomicrograph (and therefore retouched) is the translucent border as described by Leathes [284]

Our observations established one or two new points which we be lieve to be of considerable significance

Firstly all watery solutions cause an initial myelin figure outflow (i e into the aqueous phase) which is followed by a further expansion after a 5 10 minute 'delay period '

Secondly the dark field method permits interpretation of Leathes translucent border as a reciprocal phenomenon namely a penetration of the water into the lipid phase The secondary expansion phase we observed to be very dependent upon the particular ions in the watery solution If distilled water or sodium chloride (and similar salt) solu tions were used the 'secondary myelin figure outflow went on with increasing speed and vigor but the inflow of watery phase into the lipid was limited and showed little further penetration beyond that seen in the initial phase With calcium salt solutions these phase relations were reversed There was the same initial myelin figure outflow but little extension after the delay period Secondarily however the in flow of this solution into the lipid proceeded apace and without limit extending the 'translucent border throughout the lipid portion Our important conclusion was that calcium is specific in directing water into the lipid phase as is well known from the work of physical chem ists with phase relations of calcium and sodium salts in soaps and other fat water emulsions

To Leathes conclusion that myelin forms are due to surface growth and localized imbibition of water we would add that calcium ions specifically generalize the imbibition and cause a reverse mye lin figure appearance as can be seen very clearly in dark field obser vations of lecithin films

Applying these ideas to platelet (etc) alterations we conclude that the calcium has the specific role not of causing the excrescence formations (which can occur in citrate etc) but in the liquefaction of the excrescences By directing water into the altering platelets or thrombocytes calcium plays an important role in their peculiar disintegrative phenomena What about the nature of the lipid itself? In our 1945 observations [139] we were able to include some dark field observations on other phosphatides namely the purified preparations of Dr J Folch (of the Rockefeller Institute New York) which are described on p 98

The chief additional information obtained was that cephalin of the several phospholipids studied yielded myelin forms which in size and appearance e g vesicles were often so similar to the above described platelet megakarocyte and thrombocyte alterations that it seemed reasonable to regard them as identical

Actually the fundamental myelin figure phenomenon was common to most of these phosphatides in contact with watery solutions and calcium salts caused a granular deposition penetrating into the lipid and interfering with its hydrotropic dispersion into the watery phase The strength of the salt solutions had only minor effects suggestive of some membrane phenomenon in which water penetration rates are partly controlled by osmotic considerations

The inositol phosphatide (III) behaved peculiarly in that it went directly into colloidal suspension as 'chylomicra' instead of showing 'myelin forms ' This is evidently representative of the behavior of the bulk of lipoidal and lipoprotein materials of plasma

Concerning the protein components of the surfaces of platelets etc our experiments have nothing to say

(8 G) CONCLUSION

In summary then our comparisons of the dark field microscopical appearances of (a) water phospholipid films and (b) in vitro 'alterations' of platelets megakaryocytes thrombocytes and certain erythrocytes indicates the fundamental importance of myelin forms (figures)' and related phenomena at water lipid interfaces We believe these to be extremely primitive and essentially non vital phenomena of lipid rich surface membranes Not only is there an hydrotropism of the lipid material causing the appearance of surface 'excrescences' of the various types described but there is also a specific role for the calcium ion This reverses the phase relationships and directs water into the fatty phase thus contributing to the peculiar disintegrative phenomena studied Cholesterol proteins osmotic factors pH and perhaps other factors need additional study as further possible modifiers of the basic phenomena which we have described

G CLINICAL CASES WITH PLATELET PROBLEMS

FOREWORD

Our research problem 1950 55 with regard to the investigation of clinical cases with hemorrhagic and related disorders was largely planned and executed by Dr Jessica H Lewis Research Associate

and Co investigator with advice assistance and some participation by the author Invaluable technical assistance was rendered by Francis M Morgan Doris C Ferguson Iris Rudin Ann C Howe and Bertha G Jackson Since 1953 a graduate student Mr James W Fresh has made many able contributions especially to Group I studies (see below) and in the past year Miss Mary Jane Patch has been a most helpful collaborator The staffs of the University s (North Carolina) Memorial Hospital Duke Hospital the Veterans' Hospital and ORINS Hospital have cooperated with the patient material as have a number of private practitioners We are indebted to Dr Marjorie B Zucker of the N Y U College of Dentistry Physiology Department for the serum serotonin assays [519] used to assess the platelet vasoconstrictor factor (Item 17 see Tables)

In preparing the data for this thesis the author has proceeded independently and has not consulted Dr Lewis who now has her own Research Laboratory at the University of Pittsburgh Gratefully acknowledging the leading role of Dr Lewis and the individual contributions of each member of our group the author nevertheless assumes sole personal responsibility for the following presentation and analysis of the data selected

CASES represent some 86 patients and 32 normal mothers and their infants They are classified into nine groups No attempt will be made to analyze them from the clinical viewpoint Rather they represent merely a physiological contribution of analytical laboratory methods with which to approach a rational diagnosis and understanding of the clinical hemostatic problems Our groups' are chosen from this physiological viewpoint to serve the purpose of this thesis inquiry

Methods for the most part have been described on pp 53 62 and will not be further elaborated An itemization (1 30) facilitates organization and reference In the first Table (LII) the mean and range of test values are given for (A) normal healthy adults (randomized as to sex and age) (B) parturient mothers and (C) their normal newborn infants (cord bloods)

0 ± (i e trace or minor) + ++++ indicate relative degrees of (7) bleeding tendency and (12) clot retraction (in glass) Retraction was also observed in silicone (cf 11) but this will not be reported since the differences from (12) were relatively minor In group summaries the number of cases was divided for each test into the following categories:

Pos significant alteration of the test value
Neg not significantly different from the normal (norm)
? doubtfully significant
N.T.: no test performed

Results are presented in Tables LII LX

GROUP I (A) Normal Adults (B) Parturient Mothers (C) Normal Newborn Infants (Cord Bloods) ref [172] Table LII

(A) Normals were from healthy adults randomized as to sex and age They were mostly medical students staff and hospital personnel including a few colored helpers The means of the various tests were

statistically determined on significantly large groups usually more than 50 cases

(B) Mothers The 32 cases studied permitted venepuncture during parturition They all appeared healthy and well and gave no history of recent infections antibiotic or excessive salicylate therapy dietary inadequacy or gastrointestinal disturbance The obstetrical anesthesia was non contributory Pregnancy labor and the puerperium were uncomplicated by toxemia hemorrhage or thromboses

(C) Infants The 32 newborn babies yielded blood samples from the umbilical cord before it had stopped pulsating All babies were delivered without trouble were uninjured and appeared normal None developed any bleeding tendency Some mothers and some babies received vitamin K questions concerning which are answered in the cited references [172 173]

Test Results See Table LII For purposes of this thesis comment will be limited to the noting of the absence of any platelet problems in (B) and (C) All tests in this regard fall within normal (A) limits A single exception (10%) of low platelet accelerator (16) in (C) was of doubtful significance (possible lability)

The many interesting findings in other tests are fully dealt with in the cited publications and include data on another 125 newborns and most of their mothers which are not included in the present more completely studied group

GROUP II Congenital (Familial) Thrombocytopenia (Cases 1 2)
Table LIII A

Because of their unusual interest (ref [453]) these cases will be reported individually in Table LIII A

Case 1 (A B) was examined on two occasions (a) February 1 1954 before treatment and (b) April 20 1955 a few days after splenectomy In tests (a) the plat count (14) was very low (38 000) and assays for specific platelet factors (15 18) were not attempted Clot retr (12) was almost absent but prothr consumption (25) was amazingly normal The child had bleeding symptoms (7) at this time dating back to shortly after birth Bleeding time (9) was very prolonged and the T T (tourniquet test) (8) was strongly Pos

Family histories (F H) (6) will not be detailed in the present studies but were significantly Pos in Cases 1 and 2

In tests (b) after splenectomy all test values were restored to normal with the questionable exception of a 50% plat tpln (15) The patient developed no further bleeding problems in a 12 month followup

Case 2 (D D) was not a bleeder (7) despite the Pos Fam Hist (6) Nevertheless his plat count (14) was low (98 000) and tests 8 12 15 and 16 were significantly abnormal He should therefore be regarded as a potential bleeder

GROUP III: Hereditary Thrombocytopathic Purpuras Case 3 Glanzmann's Thrombasthenia Cases 4 5 Pseudohemophilia with Platelet Problems Table LIII B

Case 3 (N P) was a severe bleeder (7) with Pos Fam Hist (6) The plat count (230 000) was normal but clot retr (12) was absent and the bleeding time (9) and T T (8) were very significantly Pos Plat accel (16) was not tested and plasma proaccelerin (29) was a low normal (62%) Plat tpln (15) and ser serotonin (17) were normal (100%) Test 19 was Neg but serum antithr (20) seemed to be increased Prothr consumption (25) was normal (95%)

Conclusion This rare case seems to be one of the very few reported (ref [383]) which conform to Glanzmann's [188] 'hereditary hemorrhagic thrombasthenia '

Cases 4,5 (A C and S C mother and daughter) presented a familial (6) disorder which corresponded most nearly to the classical descriptions [484 485 486 260 262] of pseudohemophilia (ref [383 453]) Case 4 was a 52 year old white woman giving a +(+) history of bleeding tendency (7) since 19 years of age The chief manifestations were ecchymoses and a moderately severe post operative hemorrhage Bleedng time (9) and clotting times (10 11) were normal as was clot retr (12) Her plat count (14) was normal (238 000) but the plat tpln (15) significantly reduced (50%) and plat accel (16) a low normal (50%) Serot (17) N T The T T (8) was significantly +++ Prothr consumption (25) was normal (>95%) as were AHF (22) PTC(23) and other tests Plasma proaccel (29) and proconv (28) N T Fibr (21) was elevated

Case , the daughter 20 years old had also suffered +(+) ecchymotic bleedings Her plat count (14) was a low normal (104 000) and the plat tpln (15) 25% and plat accel (16) 20% unequivocally reduced Serot (17) N T Plasma proaccel (29) was normal (90%) AHF (22) likewise and probably PTC (23) 60% Prothr consumption (25) was quite normal as were other tests including a slightly questionable (71%) (recip see p 57) prothr time (26) Clot retr (12) was a satisfactory +++ Fibr (21) was elevated

Discussion In these two cases of pseudohemophilia the platelet problems were relatively minor and functional (thrombocytopathic) They must be regarded as of questionable clinical significance in a type of hereditary disorder which appears to be primarily a vascular dysfunction We had six other cases of pseudohemophilia two of hereditary telangiectasia and two with bleeding hemangiomata none of whom had any platelet problems and whose tests therefore are irrelevant to the present inquiry

Acquired deficiencie of platelet functions will be presented in the other groups The uremia cases (Group VIII) are especially interesting (p 136)

GROUP IV: Acquired Thrombocytopenia (Cases 6 30) Table LIV

These 25 cases were clinically diagnosed as follows

(A) 11 acute ITP (idiopathic thrombocytopenic purpura) of which 3 may have been due to drugs and 3 to infections namely (1) respiratory infection in a 17 mo old child (2) diphtheria in a 4½ yr old child and (3) infectious (? toxic) hepatitis in an 18 yr old white girl

(B) 7 chronic ITP including one of two months duration associated with rheumatoid arthritis in a 15 year old girl

(C) 5 were associated with anemias namely (1) two aplastic anemias (2) two macrocytic anemias one pernicious and the other occurring during pregnancy in a 22 year old woman and (3) one with Banti's syndrome

(D) 2 were associated with metastatic (bone) carcinomata in elderly males with primary prostatic malignancy

Test results are given in Table LIV in summary

Discussion Plat counts (14) ranged from 5 000 to 84 000 except for the 174 000 (at this examination December 10 1953) in a 33 year old colored woman with pernicious anemia (v C) Many showed a severe bleeding tendency (7) one was doubtful 4 N T and only one gave no bleeding history The T T (8) was Pos in 19 and questionable in 2 others who were Negroes whose dark skin interfered with the reading of this test Bleeding time (9) was Pos in 17 and questionable in one Clotting time (glass 10) was definitely prolonged in only one case but the silicone c t (11) was Pos in 10 cases This indicates the superiority of test 11 in cases with borderline clotting time increases Clot retr (12) was Pos in 17 cases None showed fibrinolysis (13) Platelet functions (15 16 17) were usually reduced pari passu with the the lowered platelet count (14) For (15) plat tpln testing 20 cases 19 were Pos and questionable in one For (16) plat accel testing 19 cases all 19 were Pos For (17) ser serotonin testing 12 cases 6 were Po Prothr consumption (25) was defective in 16 and questionable in one This is highly significant but the quantitative correspondence between platelet levels and degree of failure to utilize prothrombin showed a very poor correlation It is suggested that the quality of the platelets in terms of functional capacities is more important than mere numbers in controlling prothrombin utilization The plasma components (22 23) of this mechanism were normal except for one borderline (60%) and one ow normal (65%) PTC assays neither in the range which could be expected to influence the prothr consumption test

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GROUP V Thrombocytosis (Cases 31 33) Table LV

These three cases present different clinical disorders but show in common very elevated platelet counts (14) Two -31 and 32 had bleeding symptoms

Results are detailed in Table LV

Discussion Case 31: (R G) was a 63 year old colored man with primary adenocarcinoma of the stomach and generalized metastases At autopsy the bone marrow was found to be hyperplastic with a greatly increased number of megakaryocytes He was examined four or five times between June 23 1953 and his demise on August 27 1954 The cited tests were on the first occasion but the thrombocytosis persisted at similar levels through June 29 1954 He developed a large hematoma in the upper right arm inexplicably Except for a low plat accel (16) on January 6 1954 all tests of platelet function including clot retr (12) and prothr consumption (25) were normal Serotonin (17) was not tested however The fibrinogen (21) was a borderline (200 mg) on January 6 1954 and plasma proaccel (29) low (20%) on the same occasion These findings could go with a fibrinolytic enzyme activation which might have been overlooked in recording of a glass c t (10) *of 15 min and ++++ clot retr (12) on that occasion* We do not feel sure of the crude fibrinolysin test however unless all the clot dissolves within 24 hours

Case 32 (J L H) was a 64 year old white male long known to suffer from polycythemia rubra vera Gout was an interesting complication (ref [503]) He had received P^{32} (red cell destructive) therapy at the Veterans' Hospital and we examined him twice (March 30 1954 and April 8 1955) after such courses of treatment when he was showing some bleeding symptoms (cf Group IX) Despite the extremely high platelet counts (14) the platelet function tests (15 16) and esp (17) serotonin were significantly depressed So were the prothrombin and related factors (26 29) but not the prothr consumption (25) although this might be misleadingly calculated because of the lowered level of the original plasma prothrombin

Case 33: (E M) was a 23 year old white male leukemic whom we have excluded from Group VI solely because of the unusual thrombocytosis Serotonin (17) was not tested but the other platelet function tests were normal There was some hypoprothrombinemia (27) and prolonged prothr time (26) which might have to do with liver function This case showed no hemorrhagic symptoms

GROUP VI Leukemias with Thrombocytopenia or Thrombocytopathia Problems (Cases 34 67) Tables LVI A LVI B

Besides the thrombocytosis case (No 33 Group V) platelet problems of one kind or another presented in 34 more (i e all but 4) out of 39 cases of leukemias studied The remainder of these test data will be separated into two groups (A B) by arbitrarily dividing them at a 100 000/mm level of the platelet count

Table LVI A presents a summary of the thrombocytopenics (22 cases)

Discussion Twelve of these cases recorded bleeding symptoms (7) Platelet counts (14) ranged between 17 000 and 100 000 Platelet function tests (15 16 17) were significantly reduced in all but one or two Platelet agglutination (18) was demonstrated in 5 out of 11 tested cases The T T (8) was Pos in 5 cases and bleeding time (9) prolonged in 7 cases Silicone c t (11) was significantly prolonged in 15 cases There were 16 cases and one doubtful with defective prothrombin consumption (25) Antithrombin tests (19 20) were significantly altered in many especially if we include minor variations from the

normal controls Twelve and one questionably had a low plasma proaccelerin (29) but half again as many cases had low plat accel (16) Seven had prolonged prothr time (26) but the specific thrombin forming factors (except for the accel noted) were usually normal or nearly so

Table LVI B summarizes leukemias wi th normal (or nearly nor mal) platelet counts but with evidence of some or other deficiency of platelet functions details of which can be noted in the table Five of these 12 thrombocytopathia cases recorded bleeding problems (7)

In addition to the tests reflecting platelet functions (8 9 12 14 18 25) a prolonged silicone c t (11) might be significant in some in stances As compared with the A group's 17/22 the B group's 5/12 is a smaller proportion showing defective prothr consumption (25)

Conclusion It would appear that platelet defects whether definite thrombocytopenia or functional platelet defects (thrombocytopathias) are commonly encountered in leukemias This may very well play a leading role in the hemorrhagic tendency in leukemia Cases with abnormal values in our tests but no overt bleedings may be regarded as potentially liable to episodes of bleeding

Some less frequent reductions in other clotting factors (e g pro thrombin etc) may reflect injury to liver as well as bone marrow functions

Addendum While both acute and chronic types of the common my eloid and lymphatic leukemias are included in both sub groups the A series contained relatively more of the acute types while the B series had more chronics Included in A also were (1) one case of coincident polycythemia which is now a well recognized [503] associ ation and (2) one case which was finally diagnosed as lymphosarcoma Included in B was one case of monocytic leukemia

GROUP VII Platelet Problems in Uremia (Cases 68 78) Tables LVII A LVII B

Out of 12 uremic cases studied because of bleeding problems 11 showed various platelet anomalies These are summarized in Table LVII

(A) Cases 68 77 In this sub group of 10 cases 8 had definite bleeding problems 1 denied such and 1 lacked the necessary history (7) The findings itemized 8 12 were Neg or infrequent Platelet counts (14) were all within the normal range Quite otherwise how ever were many of the platelet function tests Thus plat tpln (15) was low in 7 cases and plat accel (16) in 7 and one doubtful The two cases tested for serotonin (17) gave significantly low values Four cases and one doubtful showed defective prothrombin consumption (25) with insufficient lowering of the tested plasma components to account for this

Conclusion We believe these data offer a new explanation for the bleeding tendency in uremics namely that their platelets while nor mal in number are functionally defective (thrombocytopathic) much as they are in many leukemics (Table LVI B) The not infrequent de fect in prothrombin consumption could be a result of this also

(P) Case 78 is of doubtful interest The one borderline (50%) value for plat accel (16) on the first of the two examinations is the only

justification for its inclusion in Table LVIII Platelet counts and other functional platelet tests were normal on the two occasions The clin ical diagnosis of chronic pyelonephritis in polycystic kidneys showed an infectious complication of an already toxemic nitrogen retention The low levels of PTC and thrombin forming factors on May 30 1955 the day before demise could have been due to liver failure We would just be guessing to blame her bleedings on some toxic disturbance to vascular integrity

GROUP VIII Platelet Problems in Cases with Plasma Clotting Diffi culties (Cases 79 85) Table LVIII

These 7 cases (including one questionable) represent a very small minority of our collected data on 52 hemophiliacs 26 PTC deficients 24 acquired hypoprothrombinemics 5 congenital hypoproconvertinemics 4 congenital hypoproaccelerinemics 8 cases with circulating inhibitors (anti AHF or anti PTC) and several others with obscure clotting prob lems The data of the 7 cases are individually analyzed in Table LVIII

(A) Cases 79 83 might be subgrouped as primarily severe liver disorders with mild systemic bleeding problems Two 80 81 had marked jaundice and four 80 83 showed no response to their hypo prothrombinemia to vitamin K Plat counts (14) in Nos 79 82 were considerably reduced (31 000 92 000) and it was questionably low (128 000) in Case 83 Functional tests were N T on Case 83 but were significantly low for (15) tpln and (16) plat accel in the four others Serum serotonin (17) was quite normal in the same cases however The T T (8) was + in Case 80 but Neg in 79 81 82 Clot retr (12) was only ++ in Case 82 but normal in 79 Lysis (see below) inter fered in the other cases The prothr consumption (25) was quite nor mal in 79 82 and N T in 83 Plasma thr c t (19) was increased in the two specimens 79 82 in which there was no interfering fibrin olysis Serum antithr (20) tested normal in 3 cases The liver dys function could account for the low levels of PTC (23) prothrombin (26 27) proconvertin (28) but not necessarily for the proaccelerin (unless the liver has a 'storage function for this component of the thrombin forming system [322 492]) Fibrinolysis was a complication which could also be explained by the severe liver injury [453] It interfered with determinations of clotting times (10 11) clot retr (12) and plasma thr c t (19) A borderline (200 mg) fibr (21) in case 83 may have been due in part (?) to the fibrin(ogen)olysin but the fibrinogen was slightly above normal in Case 81 who also showed fibrinolysin

(B) Case 84 (D H) was a 3 year old white female child who was the most severe of the 4 congenital hypoproaccelerinemia cases whom we have studied and was the only such case with unusual platelets The absence of proaccelerin (29) sufficiently explains the bleeding tendency (7) the prolonged clotting (10 11) and prothr time (26) as well as the defect (43*) in prothr consumption (25) True prothr (27) proconv (28) PTC (23) AHF (22) and fibr (21) were all normal There was no fibrinolysis (13) nor inhibitor (24) and clot retr (12) was normal The platelet count (14) was a normal 240 000 and plat tpln (15) nor mal (100*) However the platelet accel (16) was very low indeed al though it was measurable The high value (250*) for serum serotonin is interesting and the suggestion that this might be a physiological

'compensation' is an open question It was this case that most strongly influenced us toward a tentative hypothesis that the platelet accelerator is very closely related to if not identical with plasma proaccelerin

(C) Case 85 (C R) was a 70 year old white male with prostatic carcinoma and metastases His bleeding problem was primarily associated we believe with a circulating fibrinolysin (ref [453]) When first seen (January 10 1955) the plat count (14) was low (56 000) as were plat tpln (15) only 6% and plat accel (16) a barely assayable 1% Plasma proaccel (29) was also very low and fibr (21) seriously depleted Our experience with cases of active fibrinolysin supports the thesis that fibrinogen and accelerator factor are the two clotting factors most easily destroyed by the enzyme At least in clinically encountered fibrinolysin concentrations the other clotting factors are resistant If our interpretation is correct the enzyme in Case 85 is also attacking plat accel and perhaps whole platelets which could account for the anomalous platelet findings The thrombocytopenia could explain the defective prothr consumption (25) and the lack of accelerator the prolonged prothr time (26) There was a significant increase in serum antifibrinolysin (24) which follows a pattern which we and others [453] have observed in such cases The second examination seven weeks later showed the enzyme still to be present but in reduced amounts and with the same high antifibrinolysin titer (24) Platelets (14) had increased to practically normal and fibrinogen (21) and other tests (26 29) were considerably improved except for the important prothr consumption (25) which registered an unaccountable zero AHF (22) was not tested at this time and it would have been interesting to have had this perhaps vital information

GROUP IX Platelet Problems in Radiation Sickness (Case 86) Table LIX

(A) In humans our experience is confined to a single case R T (No 86) who was an elderly white male with a carcinoma of the colon which had metastasized to the liver With such a hopeless prognosis he was being given radio gold (Au¹⁹⁸) at the ORINS Hospital (Oak Ridge Tennessee) and the author in consultation with the AEC was permitted to obtain the test data given in Table LIX

Results refer only to two tests namely prothr consumption and plat counts [153] but they are very interesting On the day (1) before the radio gold injection the patient was in poor shape and the prothr consumption at 67% (23% residual in 1 hr serum) was definitely defective although the plasma prothrombin was rather high at 510 two stage (p 59) units/ml tested with a very reactive fibrinogen ('normal' = 400 450 units) It could have been evidence of benefit to the liver that the prothrombin consumption returned to normal for two weeks after the Au¹⁹⁸ injection The platelet counts were essentially normal throughout this period as they were at the start

The 19 day tests however showed the prothrombin consumption decreasing again (20% resid ~ 80% cons) while the platelets (158,000) were beginning to fall although the level at this time (on a purely numerical basis) should not have been able to account for the Pos prothr consumption test The 22 day tests showed serious deficiencies of both platelets (48 000) and prothr consumption (55% resid = 45% cons)

On the following day the patient died from a massive hemorrhage at the primary carcinoma site This could of course have been a vas cular erosion but the defective hemostatic mechanisms were certainly a fatal handicap

(B) Dog experiments

Experiment 50 Table LX

Purpose To inject dogs with Au^{198} intravenously in an effort to localize the bulk of the radiation ('hard γ and some β emissions) in the liver (which is known to sequester colloidal gold as used for these experiments) Effects on the coagulation mechanisms were to be in vestigated following such leads as appeared to be profitable and prac ticable under the rather restricted laboratory facilities made available to us due to the exactions of protective measures against the very 'hot dogs who had to be isolated in outlying buildings devoid of the central laboratory's conveniences

Methods The several test systems used were described in a pub lication [148] of these preliminary data on (2) two controls who re ceived Au^{198} colloidal suspension with an old preparation which had lost all but a trace of its radioactivity and (b) five experimental ani mals who received 20 10 10 5 and a token 1 6 millicuries/kg Au^{198} fresh from the atomic pile respectively

Results Table LX summarizes the day by day results for the prothr consumption and plat count tests on three of the animals (I II III)

Discussion Lethalities were as follows:
(I): 20 mc/kg dose: death of animal on 9th day
(II): 10 mc/kg dose: death of animal on 37th day
(III): 5 mc/kg dose: animal surviving and apparently recovered 3 months later Not illustrated are:
(IV) 10 mc/kg dose: apparent recovery after 4 weeks (according to pro cons test) but unexpected death on 83rd day
(V) 1 6 mc/kg dose: minimal effects and remained well until sacri fice on 106th day

The prothr consumption data and platelet counts given in Table LX showed some lack of correlation between the two tests Thus the prothr consumption defect appeared in dogs II and III on the 6th day at which time the plat counts were still normal Furthermore the prothr consumption test data were very different quantitatively on the 8th day in these same two animals although both platelet counts were moderately reduced and to about the same level

Autopsy Findings

Dog (I): Liver was markedly icteric hemorrhagic and necrotic Duodenum showed a section which nestled under the liver to be swol len with a massive interstitial hemorrhage Hemorrhages were wide spread and particularly noteworthy were (1) bleeding into neck from the venepuncture prick of the previous day; (2) pulmonary hemorrhages (3) petchiae in serous membranes and in mucosa of alimentary canal (5) lymph node hemorrhages

Dog (II): Liver showed acute damage reported as 'massive and complete hemorrhagic necrosis " Hemorrhages were widespread in cluding pulmonary

Dog (IV): Liver showed marked chronic damage reported as chronic cirrhosis with atrophy of liver cords engorged sinusoids and considerable hemosiderin apparently from old hemorrhage

Dog (V): Liver and other organs were normal

We are indebted to Dr G A Andrews for the autopsy reports *and to* Dr Marshall Brucer Head of the ORINS Medical Division for making these studies possible

Discussion The pathological data were supplemented with meas urements *of the residual radioactivity which confirmed the major* con centration of the Au^{198} in the liver There were however significant amounts also in the spleen lymph glands and bone marrow Hence our preliminary attempt to localize internal irradiation for a particu lar organ (e g liver) by intravenous injection of a suitably chosen preparation was not altogether *successful* What was remarkable however was the extent of liver damage which radiations thus achieved could cause without significant effects on prothrombin fi brinogen and other clotting factors

No circulating (e g heparin like) anticoagulant could be detected in our experiments

The one test which these preliminary experiments supported as showing very significant results was the prothrombin consumption *test (whether* by 2 *stage or 1 stage methods) What is of relevance* to the present thesis is the apparent lack at times of correlation with the well known thrombocytopenic effect of radiations [14] Jackson Cronkite et al [96 97 245 246] Penick et al [374] and others [477] have reported on the post irradiation thrombocytopenias and lack of effects on blood-clotting factors including AHF [374] al though causing defects in prothrombin consumption We accord great respect to these other workers particularly to Dr E P Cronkite for his work performed while a Commander in the USN including partici pation in animal experiments both at the Naval Research Laboratories (X irradiation) and in the test atomic bombings at Bikini and Eniwetok Cronkite also noted discrepancies of the two tests especially during the recovery phase post irradiation

We believe the evidence for the significant role of platelets as particularly established by Comdr Cronkite's group of workers to be most convincing However we wish to keep an open mind on the pos sibility for some role also of certain plasma factors We have plans for studying the PTC and 'Factor X' levels which we had hoped to carry out this year but the writing of this thesis has necessitated a delay which we hope to overcome in the near future

Conclusion Thrombocytopenia is a very significant correlate of the hemorrhagic problem of radiation sickness However the pro-thrombin consumption test appears to be a more sensitive index of the associated blood defect(s) and would seem to reflect with some ac curacy the severity and probable outcome of the radiation injury [148] ' The possibility of discovering radiation damage to some plasma factor or factors which work with the platelets in prothrombin utilization is still an open question Cats rendered thrombocytopenic by X irradia tion have been used to estimate platelet life span in crossed circula tion experiments [283]

PART III

INTEGRATION OF IDEAS

A FORMULATION

An integration of ideas based on the data of this thesis permits detailed formulation of the following 'working hypotheses'

1 Thromboplastic lipid There is abundant evidence for a significant and essential role in the physiological clotting process of a factor or factors of a lipoidal nature probably cephalin Its chief mode of action may be termed thromboplastic This signifies that it participates along with Ca^{++} ions in the conversion of prothrombin to thrombin in the presence of Stuart factor (R S p 27) proaccelerin and other co factors It is probable that (a) the initiation of blood clotting is dependent upon the availability of the proper form of the postulated lipoidal thromboplastin and (b) it may need some special preliminary reaction of thromboplastin generation to present it in a suitable form to the thrombin forming reaction It is questionable that tissue or plasma thromboplastins are true lipoproteins [87] Fur ther (c) there are in all likelihood certain naturally protective inhib itory mechanisms to be overcome before thrombin formation can pro ceed On these we may comment as follows

2 Natural inhibitors Heparin with its co factor is a possible inhibitor which can be demonstrated in experimental systems How ever there is considerable question as to the artificiality of such test systems and the best modern evidence (p 30) doubts that there is enough heparin in the normal circulating blood to prevent clotting Nevertheless such a mechanism could prevail locally where Mast cells are abundant in and around blood vessel walls (Jorpes [258]) Also clinical [37 448] and experimental [498 255] conditions of true heparinemia have been shown to exist Another possibility which has been particularly advanced by Tocantins et al [475] is that certain lipoidal antithromboplastins exist in the blood and are increased in cer tain states e g hemophilia [472] and post irradiation [471] Tocantins is a very careful worker but there have been criticisms of his t ch niques (e g the question of variable ionic strengths [196]) and the consensus of most American coagulationists is to minimize (though not to dismiss) the antithromboplastin idea particularly in hemophilia where the evidence for deficiency of a specific AHF or antihemophilic globulin (p 25) is convincing

3 Cephalin A number of workers have endeavored to characterize the thromboplastic lipid and its complexes and also the lipid anti thromboplastin(s) The present author has contributed in this area and current investigations are continuing with collaboration of biochemical colleagues The best evidence to date merely confirms the original

discovery of Howell (1912) and Zak (1912) The data reproduced in this thesis are particularly strong support for this The assay meth od developed in the author's laboratory is extremely sensitive and can detect the activity of thromboplastic (cephalin like) materials at dilutions that run into the millions This means that such activity may be picked up in purified materials in which the real factor is merely a trace contaminant We believe that this may explain the doubts [84 88 76] that the thromboplastic lipid is really cephalin The chief basis of such doubts is the finding of activity in alcohol or acetone soluble lipid fractions That some cephalin may find its way into such frac tions as a contaminant is a very reasonable supposition and it may very well be true that lipid solubilities are not an exact method of fractionation when dealing with the crude mixtures extracted with al cohol and ether benzene chloroform and other lipoidal solvents Hence we shall adopt as the current working hypothesis the conclu sion that the essential thromboplastic lipoids are true cephalins or ethanolamine phosphatides There is some old evidence (e g Gratia and Levene [200]) that the unsaturated fatty acids are necessary Ly socephalins are inactive [221 53 300 79] Certain synthetic cephalins containing only saturated fatty acids were found to be inactive (throm boplastically) by *ourselves pp 98 99 confirming earlier work* [202 263] A more recent synthetic cephalin prepared by Baer et al [28 29] and tested by our colleagues Dr K M Brinkhous and other mem bers of his Pathology Department (University of North Carolina) [282] was found to be very slightly active Our tests (p 99) show this to be negligible suggesting a trace impurity Other biochemical problems noted on p 99 such as possible differences between α and β cepha lins etc await further investigations

4 Lipid antithromboplastin Relatively crude cephalins ex tracted from brain and other tissues (see pp 25 71) are very satis factory for blood clotting experiments [489] They do contain some antithromboplastic lipid however which limits their use at higher concentrations (p 88) and this may be the explanation (at least in part) of the experimental finding of a cephalin optimum

One interesting new possibility is opened up by the author's most recent data (p 99) namely that antithromboplastin may not be so much a single factor as a number of lipid fractions acting together perhaps synergistically

5 Complete' ness of thromboplastin. A new way to demonstrate 'thromboplastin generation. There is no doubt that aque ous or saline tissue extracts (thrombokinase or tissue thromboplastin) in most clotting systems have a definite superiority over any currently available phospholipid preparation The author's Experiment 29 p 100 comparing Dr Quick s rabbit brain thromboplastin with the analyzed equivalent of its extracted P lipids is a particularly striking example of this Dr Seegers in personal communications reported that his highly purified prothrombins were very poorly activated by cephalin as compared with tissue thromboplastin This the present author [156] could confirm (p 90) However there was some doubt whether Seegers' thrombin forming systems were adequately provided with AcG and other necessary cofactors It may be that the excellence of cephalin in our

latest eluate (prothrombin + proconvertin) systems with added AcG was due to 'thromboplastin generation' in conjunction with the PTC and significant though small amount of AHF present (p 107 and Table XXXVII) In our best experiments (p 77) there were no differ ences in thrombin yield but only a little difference in the rates of thrombin formation Even this difference however disappeared (p 78) when more AHG (antihemophilic globulin) was added to the test system These experiments represent a new way of demonstrating the phenom enon of 'thromboplastin generation '

6 Intermediates We are not at all sure whether a specific com plex (? lipoprotein) type of complete thromboplastin is a necessary pre liminary to thrombin formation With so many factors known to par ticipate it would be difficult to avoid the surmise that a number of intermediates (see p 48) occur during the thrombin forming reaction Exact identification of these and the sequence of their appearance offers a ripe field for future harvesting Our own current conclusions favor one type of intermediary for which we believe the current experiments provide trustworthy evidence We term this the Ca cephalin protein intermediary complex The cited data indeed show (1) thrombin formation may be interrupted during its early phases by addition of decalcifying agents (oxalate citrate sequestrene or better cationic exchange resins) resulting not merely in arrest of the prothrombin activation but in a progressive inactivation to what appears to be an inert or non thrombin by product (? autoprothrombin) However when the thrombin is fully formed complete decalcification results in no significant effect on its coagulant potency Unlike the postulated inter mediary therefore true thrombin is not a calcium compound [265] Of course the decalcification may prevent prothrombin activation when performed early enough so that no Ca ions are available for the activating processes

(2) A new datum easily demonstrated (p 85) when using cephalin as the thromboplastic additive is that a similar progressive inactiva tion during the intermediate phase can be effected by using the very mild lipoidal extractive benzene Again there is no such effect when the thrombin formation has been allowed to go on to completion This we regard as evidence for participation in the intermediary of a free available mobile and loosely binding phospholipid (cephalin) We recall [149] the very poor activation by calcium alone of Howell pro thrombin which our recent analyses (p 91) show not to be lacking in AcG proconvertin PTC or AHG Such prothrombin preparations by actual lipid analyses contained significant amounts of cephalin If such amounts were added to the system in the free form i e as purified cephalin suspension they would give excellent thrombin formation

7 The cephalin availability theory This then is the basis of the author s 'cephalin availability theory ' which postulates that the phospholipid thromboplastic factor must be liberated or made available in some way from its otherwise inert lipoprotein combina tions Protein bound phosphatides are ubiquitous in both tissues and plasma even hemophilic plasma [132] How they become available to function as an essential thromboplastic factor in blood coagulation poses some very intriguing questions

8 Thromboplastic enzymes Since 1939 the author and his colleagues have performed a very large number of experiments de signed to explore the possibility that certain proteolytic enzymes may have some role in this availability of cephalin In such action we term them thromboplastic enzymes Experimentally the pancreatic protease trypsin (but not chymotrypsin) provides a considerable body of evidence as follows:

1) Trypsin addition facilitates the clotting of whole blood [107] decalcified plasmas (but not if there is too much oxalate or citrate [149]) even hemophilic blood [480] or hemophilic plasma fractions [132]

2) Trypsin acts by promoting the conversion of prothrombin to thrombin [112] but again provided that there is not too great excess of oxalate or citrate [149]

3) In the thrombin forming system trypsin acts best if there is sufficient calcium and some (but sub optimal) amount of cephalin or tissue thromboplastin Our [156] most critical Experiment 32 p 105 *indicates no effect of trypsin with adequate calcium unless a weak* thromboplastin is also added

Hence we conclude that trypsin is not thromboplastic in its own right (controverting Eagle & Harris [112]) but merely makes the phospholipid (cephalin) and sometimes even the calcium more 'avail able' for the thrombin forming reactions A possible basis for this action and to explain minor activity in the absence of sufficient Ca and P-lipid is a postulated ability of trypsin to 'disaggregate' Ca and lipid from their normally inert protein combinations

4) The antiprotease experiments (pp 113 116) clearly show loss of the thromboplastic action of trypsin parallel with removal of pro teolytic activity by suitable treatment with these anti trypsin prepara tions Controls (pp 117 119) show most of these antiproteases to be devoid of their own inhibitory effects on thrombin formation

6) The trypsin clotting of completely platelet free plasma (p 67 Table V) cannot involve any platelet potentiation This therefore calls for additional consideration of plasma sources of thromboplastic lipid which will be considered on p 145

9 The fibrinolysin question Cited evidence from the author's experience has been given to show essentially similar exper imental phenomena for the natural plasma protease fibrinolysin (plas min or tryptase [143]) However there was a most significant excep tion (p 114) namely that these thromboplastic effects in platelet potentiation experiments (which system showed them most clearly) were not abolished by antiproteases This must throw the gravest doubts [301] upon all the evidence concerning fibrinolysin and require a future investigation of some other unknown component of all our fi brinolysin preparations which could explain the findings independently of the protease proper Until this is definitively settled we withhold any commitment as to a genuine clotting role for fibrinolysin which was prematurely postulated by the author [136] in 1943

10 Platelets. The occasional success in obtaining a completely platelet free plasma which will not clot on simple recalcification even in glass (p 66 Table IV) is the best evidence that platelets are nor

mally essential for blood coagulation Three observations however still leave room for doubt Thus:

1) Why does this experiment so often fail even when the technical skill does not seem to be less than in the few experiments which suc ceed [90 472]? Is it just a matter of a little fragmented platelet mate rial eluding the experimental effort or could it be that the plasma itself sometimes contains the trace amounts of thromboplastic factor needed? Tissue thromboplastin contaminants can certainly be ruled out in the careful performance of these experiments

2) Why is it that not only platelets and tissue thromboplastin addi tives work but also and to a very significant extent cephalin (Table IV) and the proteolytic enzymes trypsin and fibrinolysin (Table V)?

A tentative answer could be that the common factor in all cases is the presence of available thromboplastic P lipid Tissue thrombo plastin must be adventitious but undoubtedly occurs in many injuries to blood vessels It appears to be quite the most effective The next best in our experiments is not platelets (much concentrated as these were) but purified cephalin Could not (experimental) trypsin disag gregate some mobile cephalin from the plasma lipoproteins as sug gested on pp 116 144? Let us defer any conclusions about fibrinolysin because of the doubts (see above) as to its purity in the preparations employed to date Pancreatic trypsin does not occur in the blood and we [293] believe the claims cited for such e g in pancreatitis are due to trypsin acting as a kinase activator of profibrinolysin

3) Finally just what is the effect of a wettable surface? It is true (p 38) that wettable surfaces and injured endothelium (p 36) have significance in the platelet alterations which ordinarily precede blood clotting However the effects noted in the platelet free system of Experiment 4 (p 66) must be on some plasma component(s) (? Hageman) Might there not be a considerable labilization of plasma lipoproteins with release of some cephalin (thromboplastic P lipid) on surface con tact? Could this not at times be the cause of the experimental failure noted in 2) above and such be an alternative explanation to that based upon postulated platelet fragments?

11 <u>The trigger mechanism</u> Correlating these speculations with those of Erkelens [118] p 51 may it not indeed be possible naturally to initiate the clotting process with thromboplastic lipid (probably cephalin) from the plasma or plasma lipoproteins? This is a very intriguing question and upon its final answer will depend the elucidation of: (a) the still mysterious trigger mechanism which physiologically initiates blood clotting when needed for defensive hemo stasis and which is obviously not in operation (at least overtly) in the circulatory blood and also (b) the true importance of the blood platelets

12 <u>Clinical disorders of platelets</u> The platelets show evidence of their important hemostatic functions in clinical bleeding disorders Only part of these functions concerns the clotting mechan ism Other hemostatic roles of the blood platelets are related to (1) their ability to aggregate and adhere to injured vascular endothelium to form cellular thrombi and (2) their participation in the provision of a vasoconstrictor factor which modern evidence (p 39) suggests is serotonin or 5 hydroxytryptamine creatinine sulfate Just how

important these two mechanisms may be is best illustrated by congen ital afibrinogenemia and some other serious clotting deficiencies The rare congenital afibrinogenemic [296] lacks the essential ingredient (fibrinogen) for clot formation That such cases survive despite an overt hemorrhagic tendency must point to the great physiological effectiveness of platelet (cellular) and vascular hemostatic mechan isms Worthy of passing mention also is the evidence of Compara tive Zoology that the lower and phylogenetically older animal species developed a thrombocyte function long before a plasma clotting system evolved [189]

13 Myelin forms In the platelet alterations (p 38) after blood is shed and which presumably also occur intravascularly after vessel injury and in thrombotic conditions the author's dark field observa tions (pp 123-127) provide very suggestive evidence for a significant role of the platelet lipids In particular it is concluded that the pecu liar platelet excrescences are simply a variety of the long-known my elin figure formations Of purified phospholipids examined with a similar microscopic technique cephalin presented appearances which often simulated the platelet alterations in very minute detail These alterative phenomena are not peculiar to platelets but could be shown (pp 127 129) also in examinations of megakaryocytes thrombocytes (of lower animals) and occasional erythrocytes undergoing stromatoly sis Furchgott [182] in 1940 also suggested the myelin figure inter pretation for the last but apparently was unfamiliar with the author's 1934 platelet observations

14 Preservation of platelet thromboplastic component Of the several factors which platelets may contribute to the blood clotting mechanism it would seem reasonable to conclude that the chief is its thromboplastic component This is at least in part extremely stable as shown by Experiment 35 (p 108) J L Tullis [479] who is directing the platelet work of the Harvard Plasma Fractionation Commission notes (op. cit p 148) During storage platelets undergo a gradual aging process A loss of fibrils (cf [124] excrescences) is one of the first morphological changes to be seen Moreover there is a concurrent loss of clot retracting ability without loss of thromboplastic activity (Tullis' italics) Using as test system the ability of preserved platelets to restore coagulability to platelet poor plasma the Harvard scientists claim remarkable pres ervation of platelets on storage The present author believes this needs to be reconsidered in view of evidence that many other platelet functions may be physiologically needed and most of them are highly labile Platelets fractionated from human donors for clinical use should be used almost immediately and even then there is evidence that their therapeutic benefits are short-lived

15 Potentiation of platelet thromboplastin The second major point about the thromboplastic action of platelets is its potentia tion by antihemophilic factor PTC etc (see p 108) Current thinking concerns a postulated 'thromboplastin generation' from some reaction (probably a series of reactions) between the platelets and the several plasma components The evidence of workers with the thromboplastin

generation test points to need not only for calcium but also for factor V (proaccelerin) etc if not for (pro)thrombin itself in thromboplastic generation The types of experiment pursued in the present thesis have not explored the possibilities of thromboplastin generation as a separate phase i e prior to the activation of prothrombin to thrombin However Experiment 40 (p 115) does show the important potentiation of platelet thromboplastic action by purified AHG and Experiment 11 (p 77) shows that cephalin can substitute for platelets in this phenomenon whereas the complete thromboplastin (tissue tpln) in sufficient amounts is not improved by the plasma component addition

Again we conclude that the phospholipid cephalin most probably is the stable factor in platelets This is a weak and incomplete thromboplastin in its own right apparently but yields a complete thromboplastin in the presence of sufficient AHG PTC etc Our eluates (Table VI) contain these plasma components especially PTC but could probably be freed if necessary from the contaminant trace of AHF This present AHF contaminant explains why our eluates and the older Howell prothrombin were so well activated by cephalin as compared say with Seegers' more purified prothrombins

Another potentiation of platelet thromboplastic action by a trace of thrombin (Experiments 36 37) is not easy to explain in our systems which are supplemented with AcG (accelerin) of the serum type i e not the precursor proaccelerin Thrombin is known [492 290] to play a role in the activation of proaccelerin to accelerin This removes a lag phase in our two stage thrombin formation tests Contrary to the claim of Seegers et al that platelet accelerator is like serum AcG Dr Lewis and the author have noted many times that it shows the lag phase in two stage clotting systems which we regard as evidence for proaccelerin (Seegers plasma type AcG) Were it not for our added accelerin therefore thrombin could be effecting a change from the precursor form (proaccelerin) to the complete accelerator (accelerin) in the platelet component It is still just possible that this is indeed the case and could account for the thrombin potentiation Consistent with this possibility is the fact which we [154] have several times demonstrated with our eluates namely increasing the AcG to an excess several times the equivalent of its strength in normal adult human plasma enhances thrombin formation with no clear cut evidence of any AcG optimum Pending more data on the exact quantities of AcG (and pro AcG) in our systems therefore we conclude that the thrombin effect might be of the nature described It is however conceivable that the mild proteolytic actions of thrombin could disaggregate phospholipid from the platelet lipoproteins as we postulate in the case of the much more powerful proteolytic enzyme trypsin Lacking concrete evidence at this time such a concept is merely a tentative suggestion which could guide future enquiry

B FINAL SUMMARIZATION

The material in this thesis supports the idea that a major role in the natural clotting mechanism must be assigned to a group of factors which collectively form the 'thromboplastic' activator(s) which in conjunction with ionized calcium convert(s) the prothrombin (precursor) into the active thrombin needed for catalyzing the conversion of

fibrinogen to fibrin clot We have advanced evidence for

1) the importance of certain lipids (? cephalin);

2) the normal need in plasma clotting for platelets;

3) the particular significance of a platelet component which has many analogies to cephalin in the thromboplastic system;

4) potentiation of the thromboplastic actions of cephalin of platelets and of tissue thromboplastin (to some extent) by various experimental additives Part of this may be explained as a 'thromboplastin generation' through co participation of certain plasmatic components (anti hemophilic globulin PTC etc) Part however may be the result of certain proteolytic enzymes particularly trypsin 'disaggregating bound forms of phospholipid from the normally unavailable lipoprotein combinations and thus rendering it 'available' for participation in the generation of thromboplastin and hence the conversion of prothrombin to thrombin;

5) possible Ca containing and lipid containing 'intermediates' in the thrombin forming reaction(s);

6) myelin figure formation as an explanation of 'alterations' of platelets and certain other formed elements such as thrombocytes megakaryocytes stromatolytic erythrocytes

7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanism;

8) the occurrence of many clinical bleeding disorders due to deficiency of platelet functions Thrombocytopen'as denote deficiency of platelet numbers and hence of the total bulk of the platelet factors available in the body Thrombocytopathias are deficiencies of specific platelet components and can be clinically significant even when the platelet count is normal Bleeding in leukemias uremias etc may often be accounted for in these terms

9) the nature and modes of action of heparin and other 'antithromboplastic' inhibitors and of some anti proteases in relation to the mechanisms discussed;

10) the 'cephalin availability theory' of the author as a useful hypothesis to explain the importance of the natural thromboplastic phospholipid Lipid release from platelet tissue or possibly plasma (Experiment 4) sources may very well be the long obscure trigger mechanism' which initiates blood coagulation

PART IV

SUPPLEMENT

THE VERSATILE TWO-STAGE METHOD FOR QUANTITATIVE STUDY OF BLOOD CLOTTING FACTORS

In the period since completion of the dissertation in the Spring of 1956 to the writing of this supplement in the Fall of 1959 the author and his collaborators have published additional papers which are listed in the continuation bibliography appended This supplement includes a selection of those portions of the recent research which are relevant to the topic of the dissertation These will deal particularly with a significant new extension of the two-stage method Originally developed as Method III" (eluate technique) for assay of prothrombin as detailed in the main text this method has now been subjected to a series of refinements and modifications whereby it can be adapted to the quantitative study of any clotting factor Such a method is somewhat empirical as regards standardization and choice of reagents of reference so that the percentage units are relative rather than absolute However the technique is reasonably simple accurate and sensitive the results being remarkably reproducible Its versatility is appreciated when by the appropriate modifications it can be used to study (a) each individual factor in the clotting system (b) inhibitors (c) effects of any additives and (d) possible intermediates in the thromboplastin and thombin-forming reactions Moreover it confirms and explains many other types of test systems and thus leads to a basic understanding of the blood coagulation mechanisms

TECHNIQUE FOR THE VERSATILE TWO STAGE

All experiments are performed at a relatively unvarying room temperature of 26 ± 3°C in mixtures at pH:7 3 buffered with imidazole NaCl Activation of prothrombin to thrombin occurs in a 5 ml mixture (incubate) of prothrombin-substrate (e g eluate) and additives all significant clotting factors in the mixture being under quantitative control

At ½-1 minute intervals 0 2 ml samples of incubate are removed and added to 0 2 ml of a standard fibrinogen and the clotting time (C T) noted

COMPLETE systems are optimal for all clotting factors whereas DEFICIENT systems lack just one factor all others being optimal or at least adequate and unvarying To the latter system the missing factor can be added quantitatively thus securing data on its effects in relation to the progressive thrombin formation

Substrate Prothrombin is usually supplied in the form of a plasma eluate When oxalated plasma is available a $BaSO_4$-citrate eluate is prepared by the method described in the main body of the dissertation We prefer this but when only citrated plasma is available we are able to secure a satisfactory substrate by $Al(OH)_3$ adsorption phosphate buffer elution and subsequent dialysis

0 1 ml of these eluates (human canine or bovine) in the routine 5 ml incubate mixture usually provides a suitable amount of prothrombin together with other eluted factors particularly Factor VII (proconvertin) Factor X (Stuart) Factor IX (PTC) and probably PTA etc Significant trace contaminants are factor V (AcG) factor VIII (AHF) Hageman factor ? etc but optima (or adequacy) of these must be assured by suitable additives when necessary

Additives Optimal calcium is regularly provided by adding 0 5 ml 0 15M $CaCl_2$ per 5 ml incubate The tissue thromboplastin which we now use instead of Soluplastin (see thesis) is obtained from acetone dried human (autopsy) brain 200 500 mg of the acetone-free dried brain powder is weighed and extracted with 10 ml saline (0 85% NaCl) for 15 min at 51-53°C (water-bath) with gentle agitation The tube is then centrifuged for 5 min at 1500 r p m and the supernatant suspension strained through gauze Routinely 20 mg of phenol is added as a preservative and the suspension is usually stable in potency for about 8 weeks in the refrigerator at 4°C The exact amount of brain powder is determined by testing several strengths of preparation in the Quick prothrombin time test on fresh normal human plasma That preparation is selected which gives an 11 sec end-point in the Quick test An optimal amount for the two-stage tests is determined and used routinely The cephalin (standard prothromboplastin) which is substituted for the tissue thromboplastin in the system modified for study of the phospholipid component and its cofactors is now routinely prepared from the same human brain powder by the Bell-Alton [38] technique The powder should give a negative Liebermann Burchardt reaction for cholesterol One gram of brain powder is extracted with successive 5-10 ml portions of reagent chloroform until a total of 50 ml of $CHCl_3$ has been used The solution is filtered through a Buchner funnel the residue dried by air jet and then finely homogenized in 50 ml saline This concentrated 2 per cent ' stock solution may be frozen stored at 20°C for as long as 12 months without loss of potency Imidazole buffered saline dilutions of the stock solution are freshly prepared or used within a few days (storage at 4°C in refrigerator) The optimal strength is carefully determined for a normal complete incubate system and rechecked from time to time in the course of a series of experiments AcG The Ware-Seegers [492] method of preparing $BaCO_3$-adsorbed beef serum for use as a source of factor V (AcG) is described in the thesis This reagent frozen-stored in 1-2 ml lots at -20°C has been found to retain its potency for several years A series of dilutions is tested to determine the optimum for each series of experiments We have recently found that this AcG preparation is also a good source of the Hageman factor and suffices for the provision of an adequate and essentially unvarying amount of this factor in most of our test systems The special problems encountered in testing for the Hageman factor will be discussed in the section dealing with that component

Other reagents These too are appropriately described in the sections dealing with the specific factor testings for which special reagents are required Generally factor deficient systems are prepared from human patients congenitally lacking the factor in question Plasma eluates provide a suitable prothrombin (etc) substrate The supernate after the adsorption when used in suitable dilution e g 1 10 1:5 0 1 ml per 5 ml incubate is often a practical way to provide accessory factors (especially AHF) still maintaining the deficiency of the factor under consideration Eluates heated to 52°C for ½-1 hour offer another means of securing deficiencies of such labile factors as V (AcG) or VIII (AHF) contaminant traces of which in fresh eluates may reduce the sensitivity of the specific tests

SPECIFIC FACTOR ASSAYS In order to adapt the test system to the specific quantitative study of any single clotting factor it is necessary to obtain the system deficient in just the factor under consideration and then to study effects of that factor addition In practice this is not difficult since it can be accomplished without the use of highly purified reagents Moreover the deficiencies need not be absolute which would often be very hard to achieve It is essential however to determine all the clotting factors in the test system and to ensure optimal or at least adequate and unvarying amounts of every one of them prior to addition of the factor to be tested All this may require considerable preliminary experimentation in order to obtain suitable reagents and test conditions The various methods including specific 1 stage tests which are described in the main thesis and elsewhere [534] are very helpful in these preliminary studies The significant results made possible by these new refinements of the two stage method will be apparent in the data to follow

PROTHROMBIN In the main thesis Experiment 10 (1956) and accompanying Table XII and Figure 14 illustrate use of the method in assay of prothrombin and Figures 10 12 show the excellent correlation of results in comparison with other methods Another similar illustrative experiment (Experiment 51) was presented at the Montreux Meetings (August 1959) and its results are reproduced in Figures 21 22 Experiment 51 brings out one or two additional points The data are for a $BaSO_4$ citrate eluate of bovine plasma and the preparation had been stored at 20°C for nearly three years Complete absence of any detectible trace of thrombin was evidenced by failure to clot fibrinogen in several days in a control test Excellent activation to thrombin however was obtained when the prothrombin containing eluate was activated by $CaCl_2$ AcG ($BaCO_3$-adsorbed beef serum) and human brain thromboplastin Thus prothrombin and such accessory factors (VII = proconvertin; X = Stuart Prower factor) as must be provided in the eluate proved to be remarkably stable under the stated conditions of frozen storage

The activation curves for the various eluate (prothrombin) dilutions shown in Figure 21 differ in shape slope and position These then indicate the activation rates which in practice are best measured by (a) the clotting time in the initial (½ or 1 min) test and (b) the incubation period required to reach the optimum The optimum is the shortest clotting time reached and this we use as a measure of

the thrombin yield as shown in Figure 22 Here the clotting-time optima from the same experimental data are plotted against the relative or 'percentage" prothrombin concentrations (eluate dilutions) The higher the original prothrombin concentration the greater is the final thrombin yield that is the shorter the clotting-time optimum A standard reference curve like this can be prepared for each substrate In the case of the normal bovine plasma eluate in this particular experiment there seems to be a logarithmic linearity within a somewhat limited range of prothrombin concentrations as shown by the inset Figure 22 Pending investigation of conditions which may be required to produce this mathematically-satisfying result it is tentatively suggested that it may depend upon absence of inhibitors together with provision of optimal (or adequate) amounts of all factors other than the prothrombin That prothrombin is the only significant variable may be deduced from the observation (Figure 21) that the optimal incubation periods were practically the same (5 6 min) in all these tests except at the 10% dilution In this older experiment accessory factors (VII X) were being diluted along with the prothrombin Clearly this would seem to make no difference provided they are over about 20% of their original level In our sensitive test system even traces of accessory factors may suffice for considerable thromboplastin- and thrombin-formation if the incubation period is sufficiently prolonged Conversely activation rates are particularly sensitive to variations in concentrations of accessory factors particularly below a certain optimum (see later) Some of these considerations may need some further study in connection with the reaction kinetics of prothrombin activation

ACTIVATORS We propose to classify the factors which participate in the activation of prothrombin to thrombin into three categories: (1) Ca-ions already covered in the thesis and concerning which we shall not say more than to re-emphasize their necessity for the test systems under present consideration (2) thromboplastic' mechanisms about which we shall have some new experimental data in the following pages; and (3) 'accessory factors: it is here we would place the newer clotting factors discovered in recent years These were reviewed in the dissertation as of 1956 The International Committee for the Standardization of the Nomenclature of Blood Clotting Factors was established in 1954 under the chairmanship of Dr Irving S Wright [549] After very careful weighing of the evidence as presented by leading workers in the field at a series of conferences including that recently (August 1959) [550] held in Montreux the Committee has now agreed upon the designation by Roman numerals of the following factors in the blood-clotting system

Factor I fibrinogen
Factor II: prothrombin
Factor III: thromboplastin (tissue)
Factor IV: calcium
Factor V: proaccelerin labile factor plasma Ac-globulin (AcG) etc
(Factor VI is unassigned)

Factor VII: proconvertin SPCA etc
Factor VIII: antihemophilic globulin AHF (antihemophilic factor) plasma thromboplastic factor A etc
Factor IX: PTC (plasma thromboplastic component) plasma thromboplastic factor B Christmas factor etc
Factor X Stuart Prower factor etc

Note that the differentiation between Stuart (factor X) and SPCA (factor VII) is now convincingly established The same is true of factors VIII (AHF) and IX (PTC) but other hemophilioid factors still need further clarification PTA (plasma thromboplastic component C etc) according to data reviewed at the Montreux Conference appears to react with Hageman factor on certain types of surfaces to yield an activator product Until the nature of these components and reactions are more clearly understood their nomenclature will remain under consideration Spaet's fourth component (plasma thromboplastic component D) [446] turned out to be merely factor IX complicated by an inhibitor problem as in our own first case of PTC-deficiency (see thesis) Tentative evidence of still newer clotting factors or possibly intermediates was also brought to the attention of the Montreux conferees particularly by Dr Koller and by Dr Alexander Our own contribution to the Montreux discussions was to present the following data on the study of the newer (and older) clotting factors by means of the versatile two stage method

FACTOR VII Figure 23 shows the activation data obtained in a specific study of Factor VII (Experiment 52) The prothrombin substrate was a $BaSO_4$-citrate eluate from oxalated plasma of a well studied congenitally hypoproconvertinemic young lady L B [535] The usual activators namely Ca AcG and tpln (human brain thromboplastin) were used for the data shown in the graphs The top (0) curve is the control for the factor VII deficient system with no other additions than those just mentioned It shows a slow activation quite satisfactory for testing effects of adding factor VII As a simple source of factor VII we used the $BaSO_4$ citrate eluate from normal human serum This of course contains some other accessory factors such as Stuart PTC etc but it was easy to show that the addition of these (as provided by a similar serum eluate from the VII deficient patient) did not change the control Thus we could standardize our system for factor VII assay using the normal serum eluate in an amount of 0 1 ml per test as our 100 'per cent' empirical assay standard This (100) and the 50 10 and 2 per cent dilutions are illustrated in the chart clearly showing the assayability of factor VII by our two-stage method Dr Alexander of course has performed similar studies on his original SPCA case material [521] In agreeing with him we merely wish to stress one point which seems to be borne out in the present series of experiments This is that the proconvertin here appears to affect only the reaction rate Ultimate thrombin yields (or optimal C T 's) were the same in all tests including the control We would not like to insist upon this point however unless we could be sure that the substrate was completely devoid of factor VII Although this case was a severe bleeder with prothrombin times usually over 60 sec it is

not ruled out that she might still have only a relative deficiency of factor VII Our system is very sensitive to even traces of accessory factors as noted above The INSET TABLE (Figure 23) gives the actual test data which show the results almost equally well by simple inspection Omitted from the curves but included in the table is a test of factor VII addition at the 200% level This seems to be slightly inhibitory at the C T endpoint Very much the same sort of thing oc curs in our test system when almost any clotting is increased beyond a certain 'optimum ' As stated in the section on PROTHROMBIN the 1 min tests and the optimal incubation periods measure the activation rate, while the shortest clotting-time reached indicates the thrombin yield In the present instance the similar optimal C T 's clearly in dicate complete or '100 per cent' thrombin yields in the control and with all factor VII additions up to its optimum

The last two horizontal lines of the inset table record an important modification of our test system This is to substitute cephalin for the usual tissue thromboplastin Our routine 'cephalin' is the Bell Alton type of phosphatide extracted by chloroform from acetone-dried human brain as described earlier We propose to term it a prothromboplastic phosphatide Another current use of the term 'prothromboplastic' [545] for accessory (or co-)factors we consider to be invalid Our use of the term indicates any phospholipid material which plays the role of an activator in the conversion of prothrombin to thrombin when it is provided with suitable cofactors in order to induce a certain type of reactivity in clotting systems This type of r activity has been termed thromboplastin generation ' [522] but some experiments (later) cause us to change the semantics of this idea somewhat Tentatively going along with the thromboplastin generation ' idea in the main thesis we stated that the above test modification (using ceph instead of tpln) converts our 2 stage system into a valuable new way to study such 'thromboplastin generation ' Of course it is necessary to have op timal (or adequate) amounts of all 'accessory' factors needed for thromboplastin generation Of these most are provided relatively readily and the one that requires most careful control is AHF Its adequacy is ensured in several ways In the present case a factor VII free AHF was supplied by adding 0 1 ml of a 1:5 dilution of patient L B's supernatant plasma after $BaSO_4$ adsorption

The extremely interesting point in this factor VII deficient system is that the absence (0) or presence (at optimal 100%) of factor VII makes no difference to the cephalin test results Our tests confirm the conclusion which others [522] have previously arrived at by the thromboplastin generation test and other methods namely that factor VII is not needed for thromboplastin generation

HAGEMAN FACTOR Figure 24 shows data on the Hageman Factor (Experiment 53) Here the data are just the reverse of the factor VII results The inset table shows that Hageman factor is without influ ence on prothrombin activation by tissue thromboplastin However the curves clearly indicate that this factor is very much needed for the activation by cephalin that is for the generation of thromboplastin The control (0) without added Hageman factor (see below) was very poorly activated and successive increments 1 10 100 units of added Hageman gave a nice family of curves for Hageman factor assay

In Experiment 53 the substrate was a $BaSO_4$ citrate eluate from oxalated plasma of one of our [534] markedly Hageman deficient cases (L C) This experiment presented some technical problems Our ordinary AcG ($BaCO_3$ treated beef serum [492]) was found to contain Hageman but we [537] were able to remove it completely by continuous flow paper curtain electrophoresis at the expense of some loss in potency and stability of the AcG preparation Dr R H Wagner [548] (of our Pathology Department) generously provided us with some of the purified AHF preparations which he had been fractionating from various animal bloods The purified AHF used in the present experiments was quite satisfactory and devoid of Hageman according to the control (0) here and by all our usual tests The Hageman preparation was one which we prepared from outdated blood bank plasma following the directions kindly supplied to us by Dr Ratnoff [543] We have good data also on those fractions from our [534] paper curtain electro phoresis in which Hageman was the only clotting factor demonstrable

STUART FACTOR Figure 25 shows data for the Stuart Factor [298 533] (Experiment 54) The substrate was a dialyzed $Al(OH)_3$ phosphate eluate from citrated plasma of a severe congenital Stuart deficient (A G) The AHF was patient A G 's Stuart free supernate after the plasma adsorption The Stuart factor preparation was obtained from normal human plasma by continuous flow paper curtain electrophore sis by our associate Dr C L Johnston [534] This was an excellent fraction rich in Stuart with no factor VII or any other clotting factor according to an exhaustive series of tests It is now well known that Stuart factor differs from factor VII in being required for thrombo plastin generation as well as for conversion of prothrombin to throm bin [533] As expected therefore it turned out to be a significant variable in both types of our test system Just a few of the activation curves are included in the composite chart of Figure 25 The initial activations in the Stuart deficient systems were often so slow that the ordinate in the top half of the chart was changed to minutes and the dotted lines indicate the break in the scale These curves show the very poor activation in the two Stuart free controls with either type of thromboplastic agent The thromboplastin curves are DASH lines and include just one with optimal 1 1 Stuart addition A 1:10 and 1 1 Stuart addition are shown in the cephalin series Particularly note that with optimal (1:1) Stuart the activation with cephalin is just as good as with thromboplastin (in fact the test times were point by point identical) except for the first minute This last is an illustra tion of the initial delay in the phospholipid activated system which leads us to conclude that it takes a minute or two for (so called) 'thromboplastin generation' from the lipid to reach the equivalent of tissue thromboplastin in our test system

OTHER ACCESSORY FACTORS Without encumbering this presenta tion with the experimental details we would like to state that we have good preliminary data analogous to the above for modified two stage assays of factor V factor VIII and factor IX We have not yet had the opportunity to study a satisfactory case of PTA deficiency but believe this factor should be equally amenable to quantitation by the cephalin two stage method

THROMBOPLASTIN FACTORS Figure 26 (Experiment 55) shows typical prothrombin activation curves using normal canine plasma eluate to test (I II) optimal cephalin or (III IV) optimal tissue throm boplastin respectively without or with factor VIII (AHF) all other factors being normal The cephalin activation without AHF (I) is pro longed and deficient With AHF (II) it is complete in a short time (3 min) but is still slow initially because of the delay required for 'thromboplastin generation ' The tpln activation is fast starting and rapidly completed (2-3 min) with no difference whether AHF is ab sent (III) or present (IV) Thus our two stage is a valuable way to distinguish between the two types of thromboplastic agents which il lustrate what Dr Brinkhous and colleagues [282] designate by the operational terms "partial and ' complete' thromboplastins recog nizing of course that both act only in conjunction with several other cofactors

Table LXI summarizes results of similar tests (Experiment 56) *on several thromboplastic materials all tested at their experimentally* determined optimal concentrations with and without AHF The per- centage thrombin yield values are computed in the usual way The rate data are replaced by qualitative descriptions which fairly indicate the basis for comparisons Thus the alternatives 'fast' or 'slow' refer to the initial reaction rates while 'short' or 'long' refer to the optimal incubation periods The differences between tissue thromboplastin (1) and cephalin (2) which were defined in Experiment 55 are restated Test (3) is with Sbp, 'Inosithin ' a crude alcohol-insoluble fraction of 'Asolectin' (soybean inositol phosphatide) [527 528] *it gave evidence of contamination with some inhibitor Test (4) is with Eap, a synthetic L-α-dioleoyl ethanolamine phosphatide recently prepared by Dr E Baer of Toronto *its potency was weak but significant and it did better in another type of test system (with Stypven) which will be described later Test (5) is with Plat human washed platelets obtained with the ADL Cohn Fractionator [546] they probably retained a little AHF contam inant Test (6) is with Rbc human erythrocyte hemolysate prepared by Dr Quick's [532] method Test (7) is the very satisfactory control Clearly all these agents 3 6 behave like cephalin with significant improvement of their activator function on adding AHF In this they are all unlike tissue thromboplastin and therefore fit the subcategory of partial thromboplastins or as we prefer to designate them pro- thromboplastic factors

Experiment 57 deals with prothrombin (canine eluate) activations by (I) optimal cephalin (II) tissue thromboplastin at 1 10 optimal strength and (III) a mixture of I and II AHF was added in series (A) and omitted in (B) The essential data are emphasized in the simpli- fied charts of Figure 27 which merely indicate progression from the initial (½ min) clotting times to the optima shown details of the in tervening curves being omitted Clearly the mixtures III were better than with either thromboplastic agent alone If we had merely done experiment (A) with adequate AHF we could have explained the result simply as the additive effect of extra thromboplastin 'generated' from the phospholipid But the synergism was equally good in (B) without the AHF when the cephalin alone (B II) was very inadequate because of the deficiency in so called 'thromboplastin generation ' How then can we explain this result? Before attempting to answer this question we shall present another line of experimental enquiry

STYPVEN The following experiments modify our two stage further by introducing the artificial additive Stypven (Russell's viper venom) The reason for this follows We were reinvestigating the 2 stage method as a suitable procedure for assaying thromboplastin In the usual system composed of normal plasma eluate adequate in Stuart (X) and factor VII as well as prothrombin and with optimal added Ca and AcG various tissue thromboplastins can be compared with our standard human brain preparation which is a satisfactory standard although it dilutes out rather rapidly Our cephalin likewise seemed to be a good practical standard for assaying prothromboplastins For such assays however the system needs in addition to the above factors (but excepting factor VII) adequate and controlled amounts of AHF PTC PTA Hageman ? etc The most significant of these variables is AHF (factor VIII) which is difficult to prepare preserve and standardize In looking for a way to circumvent this we tried Stypven This now resulted in a somewhat different bioassay system but one which fulfilled excellently the requirements for prothromboplastin assay as shown by the following experiments

Experiment 58 is the new Stypven modified two stage using canine plasma eluate optimal AcG and calcium and a series of dilution of brain cephalin (Ceph) as the standard Dr Baer's synthetic dioleoyl cephalin (Eap) was the 'unknown ' tested at two dilutions Table LXII gives the results citing the clotting times after the 1 min and the optimal incubation periods Tests 1 7 are the standard cephalin dilutions and test 8 is the control Tests 9 and 10 are the two 'unknowns ' which check well and indicate the 1:1 Eap to be equivalent to about 3% the potency of the 'standard' cephalin Without attempting to assess possible losses or inactive material but on a simple weight for weight basis the Eap potency was slightly less than 1/400th that of the Ceph Although this is weak it is by no means inconsiderable The preparation of 1 2 dimyristoyl phosphatidyl α ethanolamine mentioned in the section on synthetic phosphatides in the main dissertation was also retested in the new system and found to be inert It would appear that Dr Baer by introducing unsaturated fatty acids into the cephalin molecule has now achieved a genuine synthesis of a prothromboplastic phosphatide It could be merely a matter of the best fatty acids and physico chemical homogenization to give us a really potent preparation

The demonstrable activity of brain cephalin at a dilution of 3 in 10 000 000 (test 7) may indicate a fact of great physiological significance namely that an extremely minute amount of prothromboplastic lipid can suffice This may indeed be the normal trigger mechanism' for the initiation of blood clotting

Experiment 59 was designed to explore the mode of action of Stypven by testing in a series of factor deficient incubates

REAGENTS Prothrombin containing substrates (eluates) were from normal (dog) and human congenital deficiencies of proconvertin (factor VII) case L B of Stuart (factor X) case A G or of Hageman L C (see earlier) The PTC (factor IX) deficient was the $Al(OH)_3$ phosphate eluate from a severe case (A Y) of Christmas disease AHF (factor VIII) and AcG (factor V) deficiencies were secured experimentally by heating the normal canine eluate to 52°C for one hour The AcG AHF (purified) Stypven (Burroughs Wellcome) and throm

boplastic agents were the same as in the preceding experiments Stuart (factor X) preparations were (A) the bovine "proconvertin ' containing both Stuart and factor VII which is described in the main dissertation and (B) Stuart factor free from all other clotting factors purified by continuous flow paper curtain electrophoresis [534] as in Experiment 54 The Hageman factor preparation was described in Experiment 53

TEST SERIES (I) Using normal (dog) eluate and optimal Ca cepha lin and AcG the optimal concentration of Stypven was first determined This was 0 2 ml of 1:10 000 venom solution per usual 5 ml incubate This amount was used in all the subsequent experiments

TEST SERIES (II) included the following additions to normal eluate (1) Ca AcG AHF (no Stypven or ceph); (2) Ceph AcG AHF Stypven (NO Ca); (3) Ca Stypven (NO ceph AcG or AHF); (4) Ca Stypven AcG (NO ceph or AHF); (5) Ca Stypven AHF (NO ceph or AcG); (6) Ca Stypven AcG AHF (NO ceph) No significant thrombin for mation occurred in any of these controls thus proving the necessity for Ca ions and for prothromboplastic phosphatide Incidentally they show also that the lipid must be extrinsic and not come from any of the protein materials tested

TEST SERIES (III) is shown in Table LXIII recording the deficien cies the additives and the initial (½-1 min) and optimal clotting test results The summarized conclusion is that Stypven with cephalin and optimal Ca AcG and Stuart factor (adequate in all these eluates) gives rapid and complete thrombin formation even when the system is deficient in (1) AHF (2) PTC (3) Hageman or (5) factor VII Since our usual bovine AcG (Experiment 53) contains Hageman factor the supernatant $BaSO_4$ plasma (diluted 1:10) from L C was substituted in test 4 in order to supply AcG without factor X (Hageman) It worked almost as well as the usual AcG (test 3) The AcG-deficiency obtained by heating the normal canine eluate according to test 6 was no abso lute but the Stypven test results were poor compared with the unheated (AcG+) AcG supplemented eluate in test 14 In test 7 Stypven was omitted and the hemophilic (AHF-) system showed the usual poor activation with Ca ceph AcG In tests 8 11 the various deficient eluates all gave very little thrombin formation when Stypven was used without cephalin In test 12 with Stypven plus cephalin there was deficiency of AcG as well as Hageman In test 13 a potent AcG free Hageman preparation (see Experiment 53) was added (Hag +) with out improvement This confirms the need for AcG in the Stypven-cephalin test system

TEST SERIES (IV) was a special study of relationships of thrombin formation to factor X (Stuart) the substrate being eluate from case A G (Experiment 54) as noted under Reagents All additions were optimal for the preparations used Table LXIV records the experi mental data *including computations of the percentage thrombin yields* Stypven alone test 1 and when Stuart factor was added test 2 proved ineffective With Stypven plus cephalin test 3 the 52 per cent throm-bin yield was inadequate compared to results with added Stuart factor preparation A, test 4 or B test 5 Tissue thromboplastin showed its

usual (Experiment 54) inadequacy in the Stuart deficient system test 8 and adding Stypven test 6 did nothing to the thrombin yield but may have caused a slight improvement in the activation rate Again restoration of Stuart factor test 7 returned this system to normality both as to rate and thrombin yield This could also be accomplished without the Stypven tests 9 10 when either Stuart preparation was added Cephalin with AHF (test 11) showed very slow and incomplete activation and this could be normalized by Stuart factor addition without any Stypven (test 12) Besides confirming previous (Experiment 54) two stage test findings on the Stuart factor these new results clearly indicate that Stuart factor is needed for the modified test system employing Stypven plus cephalin

DISCUSSION These experiments show in the clearest possible manner that Russell's viper venom (Stypven) is not itself a thromboplastin nor prothromboplastin (by our definition) but is a special type of accessory factor acting in its own right It is specifically accessory to prothromboplastic phosphatide (e g cephalin) and needs also Ca AcG and Stuart factor in order to convert prothrombin into thrombin Stypven does not act like factor VII or any other physiological accessory factor The venom activates a factor VII deficient system not because it replaces proconvertin but simply because its reactions with the phosphatide and other activator components do not require factor VII Only tissue thromboplastin needs factor VII and Stypven does not improve tissue thromboplastic activity significantly in our two stage test system as shown in the next experiment

Experiment 60 These are our usual activations of normal eluate (canine) with optimal Ca and AcG but varying the concentration of brain tissue thromboplastin (100% = optimal) with or without the addition of optimal Stypven The ½ min and optimal clotting times are recorded in the simplified presentation of Figure 28 which emphasizes the essential findings The addition of Stypven in no case changed the optimal clotting time or optimal incubation period to any significant extent There may possibly have been a slight improvement in the initial (½ min) activation rates with the Stypven addition but the data are not sufficiently convincing on this minor point All in all we fail to find any real evidence that Stypven works with tissue thromboplastin

Experiment 61 was designed to show that the other prothromboplastins which so clearly resembled brain cephalin in the tests of Experiment 56 could all substitute for the routine lipid in the Stypven modified two stage system The data given in Table LXV are sufficiently explanatory it being quite evident that all the tested 'prothromboplastic' preparations gave excellent and similar (final) thrombin yields in this test system Even the synthetic Eap was very satisfactory when the incubation period was sufficiently prolonged

TRYPSIN and THROMBIN Further diversity of activator systems can be shown experimentally We have some good preliminary data to demonstrate that trypsin or a trace of thrombin can act as accessory factors in our cephalin modified two stage system These agents act only in the presence of Ca ions and prothromboplastic lipid (cephalin) They also require a group of other cofactors including V

VII VIII IX and X but not Hageman in the thromboplastin and thrombin-forming incubation systems

FINAL INTEGRATION OF IDEAS

These new experimental data definitely contribute further evidence in support of our major thesis concerning the key role of prothrombo plastic phosphatide (probably cephalin) in the early phases of prothrom bin activation The facts (a) that tissue thromboplastin certain ceph alins and a variety of preparations known to be rich in phosphatides all serve the common purpose of assisting in the activation of pro thrombin to thrombin together with (b) the significant diversity of the groups of 'accessory' factors which can work with them to achieve this result particularly in the prothromboplastic test systems seem to us to require a fundamental explanation This we suggest should not be in terms of factor entities as such but rather in terms of the dynamic processes involved In order to emphasize these processes and the common features of diversely constituted test systems we suggest that the substantive term thromboplastin be avoided and re placed by the dynamic concept more appropriately conveyed by the adjectival thromboplastic mechanisms The further descriptive characterization prothromboplastic applicable to the role of certain phosphatides (e g cephalin) seems fully justified by the experimental evidence Our main thesis defends the working hypothesis that the availability' of such prothromboplastic factors constitutes 'the trig ger mechanism ' and determines the subsequent series of intermediary reactions essential for the formation of active thrombin and hence for blood clotting In recent years the concept of ' thromboplastin gener ation has gained wide acceptance It may seem apparent to many readers that we have been talking around the subject of 'thromboplas tin generation' and that as we ourselves have indicated our new uses of the two stage test may be regarded merely as another way to demonstrate the phenomena of this so-called thromboplastin or 'intrinsic' thromboplastin generation It may be a question of semantics but we believe that some of our data require a different concept It is particularly difficult to explain Experiment 57 on the synergistic action of cephalin with suboptimal tissue thromboplastin in the absence of factors essential for the recognized 'thromboplastin generation ' Then again the 'intrinsic' thromboplastin shows differences from 'extrinsic' (tissue) thromboplastin in such things as factor VII need and in significance for clinical hemostasis the complete answers to which have not yet been worked out We propose therefore a new clotting theory based on the ideas of our main thesis but carrying the concepts some what further We suggest the hypothesis of phospholipid transfer mechanisms as basic to the coagulation reactions This working hy pothesis envisions prothromboplastic phosphatide made available at the very outset of the blood changes that lead to clotting Whether this be primarily from platelet alterations (fully discussed in the dissertation) from surface-activated plasma factors (bringing in the re cently discovered interactions involving Hageman factor and PTA) [550] or biochemical changes in certain lipids and plasma lipoproteins (as the very recent work of Dr Esnough [524] is beginning to suggest) and what it is that coordinates these processes need further

elucidation Many accessory factors are clearly involved and there must be an ordered sequence of intermediary reactions It does seem reasonable to postulate phospholipid changes and transfers to a series of proteins through such a series of intermediate reactions Pending the positive identification and perhaps isolation of such postulated intermediates we propose to adhere to a dynamic concept visualizing (a) trigger release of prothromboplastic lipid (b) a series of lipid and lipoprotein reactions (lipid transfer ?) and (c) final entry of prothrombin into these reactions with resulting alteration of its prote n structure to yield the active enzyme thrombin We have discussed at some length in the thesis the possible significance of certain proteolytic phenomena What may turn out to be of more fundamental significance is the now demonstrated polypeptide-ester (e g TAME) splitting ac tivities possessed in common by thrombin Stypven [547] trypsin and fibrinolysin (= plasmin) An unexplored field until most recently is the possible relation of lipid altering enzymes (lecithinase ?) to coagulation and it will be interesting to watch for these developments in the future

While many fundamental problems have still to be solved in the complex field of blood coagulation our dissertation will conclude with a note of optimism Much has been learned in the past and greatly added to in recent years Rationalization of the majority of clinical problems has been advanced by the discoveries of the newer clotting factors Practical tests for these are now available and as techniques are improved factor isolation should become increasingly practical and hopefully of therapeutic application The theoretical goal of basic understanding may be more difficult but many researchers are contributing toward it Among the vast literature accumulat ing in this field due place may perhaps be found for the contents of this compendium of experiments and thinking in the achievement of which the author's debt to his colleagues and associates is herewith gratefully acknowledged Let us paraphrase the words of Robert Louis Stevenson and conclude: ' To travel together joyfully is better than to arrive and the true success is to labour

APPENDIX I

FIGURES AND ILLUSTRATIONS

FIGURES AND ILLUSTRATIONS

Figure 1 SKELETON SCHEME OF CLOTTING MECHANISM

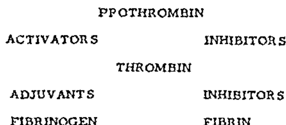


Figure 2 HEMOSTATIC AND CLOTTING MECHANISMS

INJURY HEMOSTASIS (a) Vascular factors
(b) Cell thrombus }
(c) Fibrin clot } THROMBOSIS

SURFACE ALTERATION of (a) Endothelium
(b) Platelets etc
(c) Plasma components

INTERMEDIARY REACTIONS THROMBOPLASTIN- AND THROMBIN FORMATION
Multicomponent (stepwise) reactions

PROTHROMBIN ACTIVATORS (a) Ca-ions
(b) THROMBOPLASTIC FACTORS
(c) ACCESSORY FACTORS incl
V VII VIII IX X PTA Hageman
? etc

PRE ACTIVATIONS (a) Thromboplastin generation'
(b) Proaccelerin (+ thrombin) → accelerin
(c) Proconvertin (+ Ca tpln) → convertin
(d) PTA + Hageman + Surface → 'active product

CLOTTING PROPER FIBRINOGEN → FIBRIN
(a) THROMBIC enzyme initiates
(b) Many factors modify (see text)

CLOT (THROMBUS) RESOLUTION (a) clot retraction
(b) fibrinolysis
(c) fibrosis

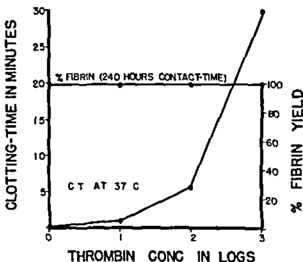

EFFECTS OF CHANGE IN THROMBIN CONCENTRATION ON THE SECOND PHASE OF CLOTTING
CLOTTING-TIME IN MINUTES
% FIBRIN (240 HOURS CONTACT-TIME)
C T AT 37 C
% FIBRIN YIELD
THROMBIN CONC IN LOGS
30
25
20
15
10
5
0
1
2
3
100
80
60
40
20

FIGURE 3

Figure 1 SKELETON SCHEME OF CLOTTING MECHANISM

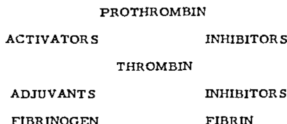


Figure 2 HEMOSTATIC AND CLOTTING MECHANISMS

INJURY HEMOSTASIS (a) Vascular factors
(b) Cell-thrombus } THROMBOSIS
(c) Fibrin clot }

SURFACE ALTERATION of (a) Endothelium
(b) Platelets etc
(c) Plasma components

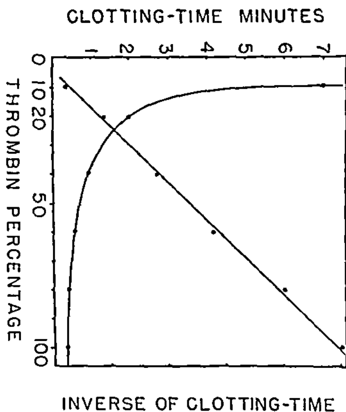
INTERMEDIARY REACTIONS THROMBOPLASTIN- AND THROMBIN FORMATION
Multicomponent (stepwise) reactions

PROTHROMBIN ACTIVATORS: (a) Ca ions
(b) THROMBOPLASTIC FACTORS
(c) ACCESSORY FACTORS incl V VII VIII IX X PTA Hageman ? etc

PRE-ACTIVATIONS: (a) Thromboplastin generation'
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(b) Many factors modify (see text)

CLOT (THROMBUS) RESOLUTION (a) clot retraction
(b) fibrinolysis
(c) fibrosis


CLOTTING-TIME MINUTES
1
2
3
4
5
6
7
0
10
20
50
100
THROMBIN PERCENTAGE
INVERSE OF CLOTTING-TIME

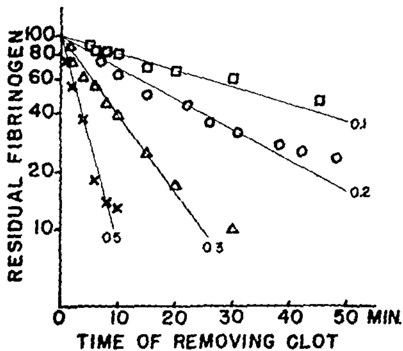
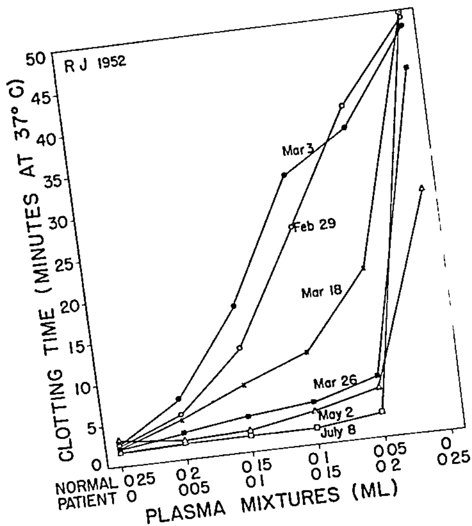

RESIDUAL FIBRINOGEN
100
80
60
40
20
10
0.1
0.2
0.3
0.5
0
10
20
30
40
50 MIN.
TIME OF REMOVING CLOT

FIGURE 4


R J 1952
CLOTTING TIME (MINUTES AT 37° C)
50
45
40
35
30
25
20
15
10
5
0
Mar 3
Feb 29
Mar 18
Mar 26
May 2
July 8
NORMAL 0 25 0 2 0 15 0 1 0 05 0
PATIENT 0 0 05 0 1 0 15 0 2 0 25
PLASMA MIXTURES (ML)

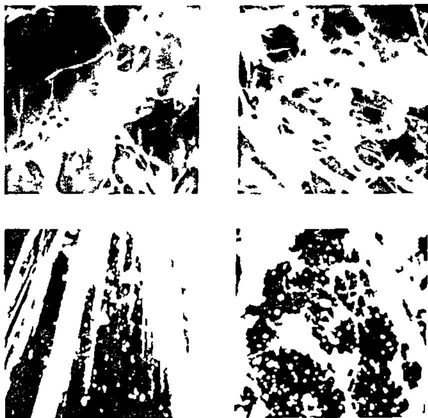


FIGURE 6

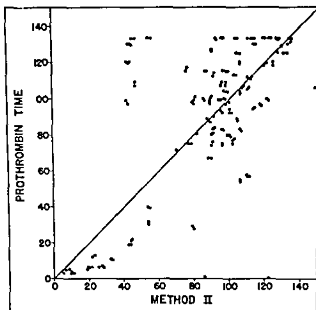
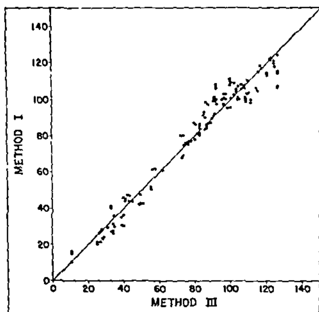

PROTHROMBIN TIME
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
METHOD II



FIGURE 8


METHOD I
METHOD III
0
20
40
60
80
100
120
140

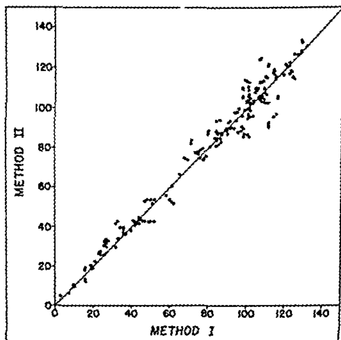
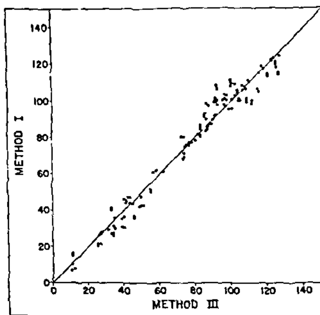

140
120
100
80
60
40
20
0
0 20 40 60 80 100 120 140
METHOD II
METHOD I

FIGURE 10


METHOD I
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
METHOD III

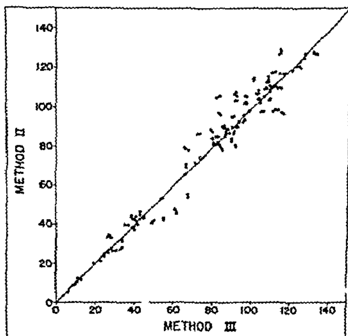
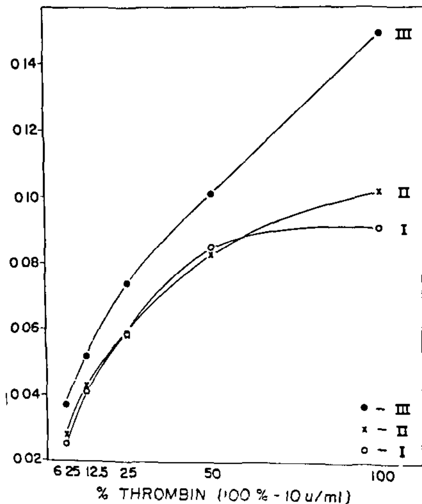
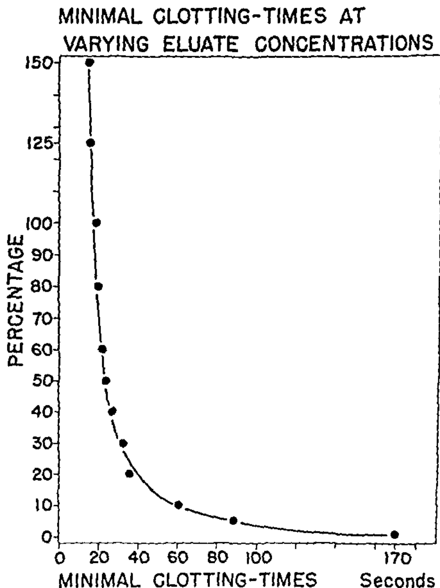

METHOD II
METHOD III
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140

FIGURE 12

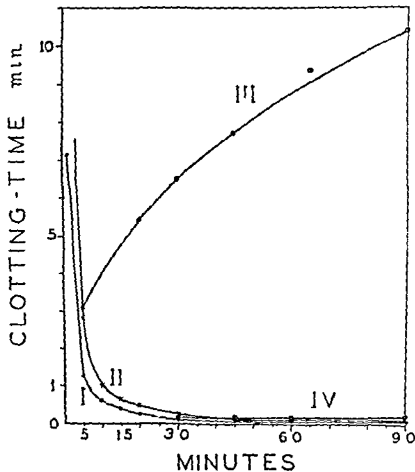


$\frac{1}{CT}$ AT VARYING THROMBIN CONCENTRATIONS
0.14
0.12
0.10
0.08
0.06
0.04
0.02
III
II
I
● – III
x – II
o – I
6.25
12.5
25
50
100
% THROMBIN (100 % - 10 u/ml)



MINIMAL CLOTTING-TIMES AT
VARYING ELUATE CONCENTRATIONS
PERCENTAGE
150
125
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
170
MINIMAL CLOTTING-TIMES
Seconds

FIGURE 14


OXALATION
CLOTTING-TIME min
10
5
1
0
III
II
I
IV
5
15
30
60
90
MINUTES

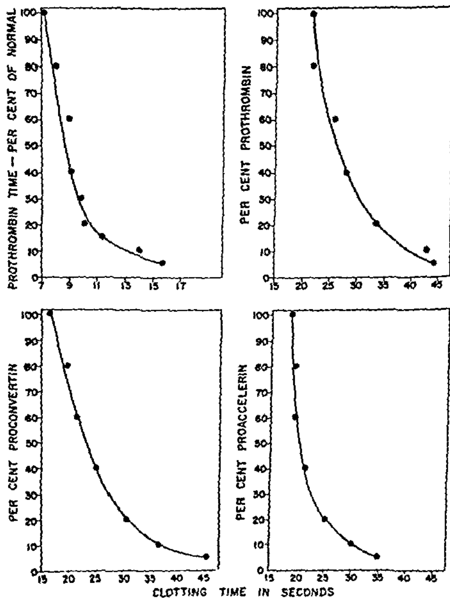
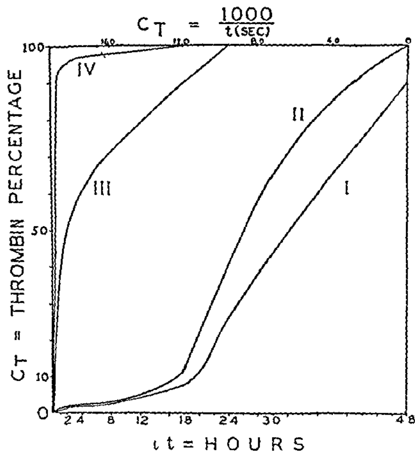

PROTHROMBIN TIME — PER CENT OF NORMAL
PER CENT PROTHROMBIN
PER CENT PROCONVERTIN
PER CENT PROACCELERIN
CLOTTING TIME IN SECONDS

FIGURE 16



$C_T = \frac{1000}{t(SEC)}$
160
120
80
40
0
100
50
10
0
IV
III
II
I
CT = THROMBIN PERCENTAGE
2 4 8 12 18 24 30 48
t = HOURS

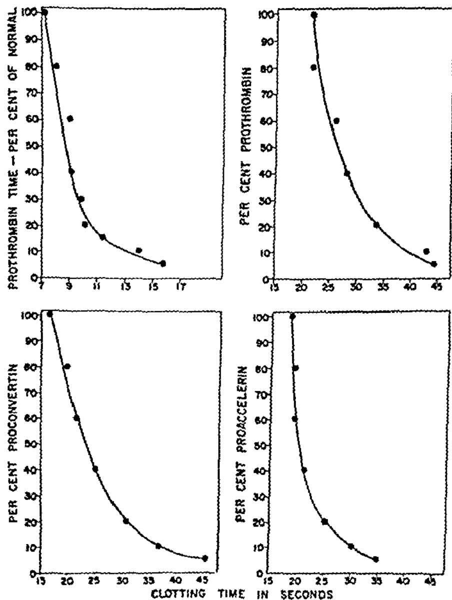

PROTHROMBIN TIME — PER CENT OF NORMAL
PER CENT PROTHROMBIN
PER CENT PROCONVERTIN
PER CENT PROACCELERIN
CLOTTING TIME IN SECONDS

FIGURE 16


5


7


6


8

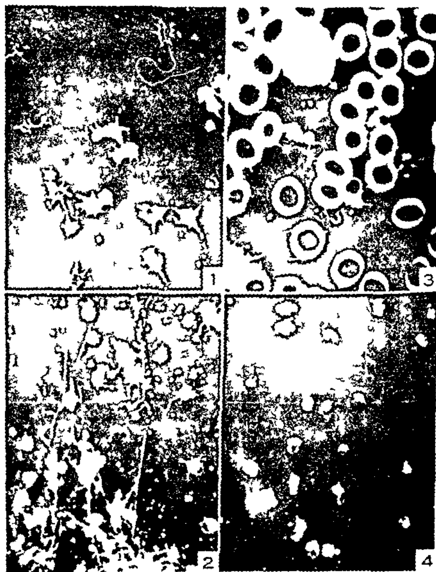
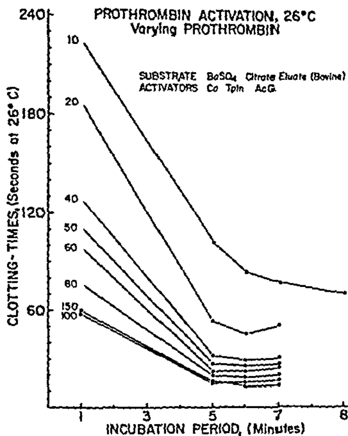
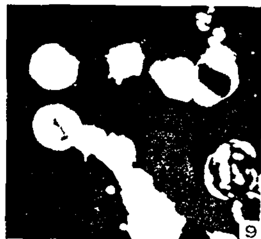


FIGURE 18



PROTHROMBIN ACTIVATION, 26°C
Varying PROTHROMBIN
SUBSTRATE BaSO4 Citrate Eluate (Bovine)
ACTIVATORS Ca Tpln AcG
CLOTTING-TIMES, (Seconds at 26° C)
240
180
120
60
10
20
40
50
60
80
150
100
1
3
5
7
8
INCUBATION PERIOD, (Minutes)

FIGURE 21









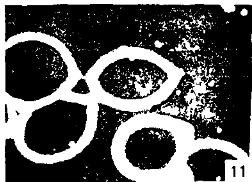
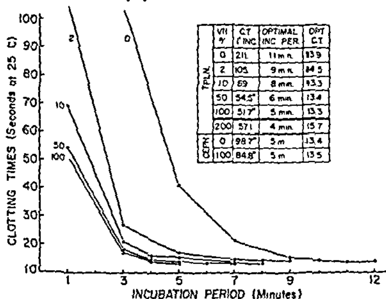
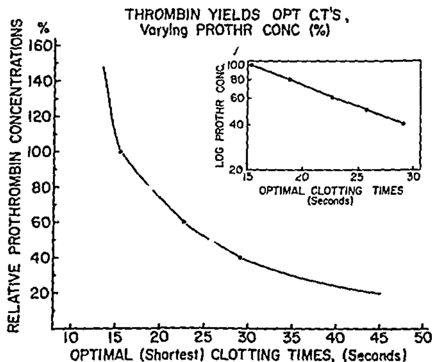


FIGURE 20



	VII γ	CT 1' INC	OPTIMAL INC PER.	OPT CT
TPLN.	0	211	11 min.	13.9
	2	105	9 min.	14.5
	10	69	8 min.	13.3
	50	54.5"	6 min.	13.4
	100	51.7"	5 min.	13.3
	200	57.1	4 min.	15.7
CEPH.	0	98.7"	5 m	13.4
	100	84.8"	5 m	13.5

FIGURE 23


THROMBIN YIELDS OPT C.T'S,
Varying PROTHR CONC (%)
RELATIVE PROTHROMBIN CONCENTRATIONS %
160
140
120
100
80
60
40
20
10 15 20 25 30 35 40 45 50
OPTIMAL (Shortest) CLOTTING TIMES, (Seconds)
LOG PROTHR CONC
100
80
60
40
20
15 20 25 30
OPTIMAL CLOTTING TIMES
(Seconds)

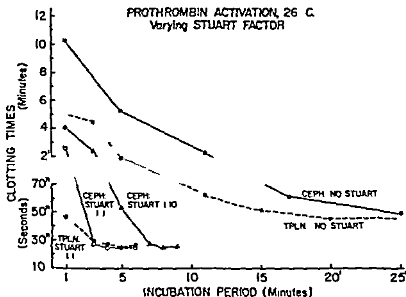
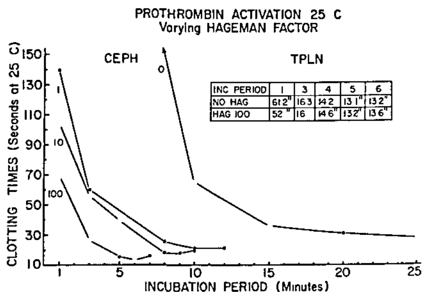

PROTHROMBIN ACTIVATION, 26 C.
Varying STUART FACTOR
CLOTTING TIMES
(Minutes)
(Seconds)
12
10
8
6
4
2'
70"
50"
30"
10
CEPH: STUART 1:1
CEPH: STUART 1:10
TPLN. STUART 1:1
CEPH NO STUART
TPLN NO STUART
1
5
10
15
20'
25'
INCUBATION PERIOD (Minutes)

FIGURE 25



INC PERIOD	1	3	4	5	6
NO HAG	61 2"	16 3	14 2	13 1"	13 2"
HAG 100	52 "	16	14 6"	13 2"	13 6"

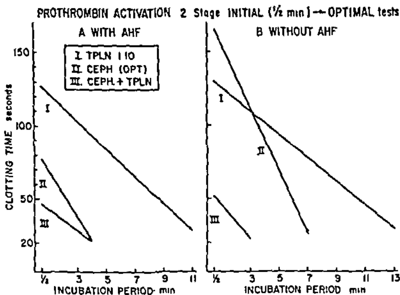
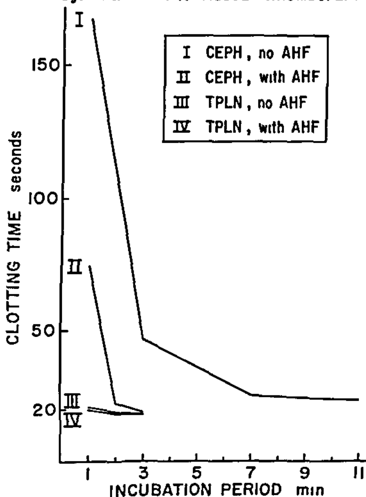

PROTHROMBIN ACTIVATION 2 Stage INITIAL (½ min) → OPTIMAL tests
A WITH AHF
B WITHOUT AHF
I. TPLN 1 10
II. CEPH (OPT)
III. CEPH.+TPLN
CLOTTING TIME seconds
150
100
50
20
I
II
III
½
3
5
7
9
11
13
INCUBATION PERIOD min

FIGURE 27


PROTHROMBIN ACTIVATION CURVES
2-Stage CEPHALIN vs TISSUE THROMBOPLASTIN
I CEPH, no AHF
II CEPH, with AHF
III TPLN, no AHF
IV TPLN, with AHF
CLOTTING TIME seconds
150
100
50
20
I
II
III
IV
1
3
5
7
9
11
INCUBATION PERIOD min

APPENDIX II

TABLES OF EXPERIMENTAL DATA

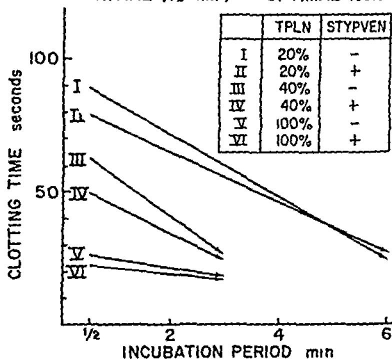

PROTHROMBIN ACTIVATION 2-Stage
INITIAL (½ min) → OPTIMAL tests
TPLN
STYPVEN
I 20% −
II 20% +
III 40% −
IV 40% +
V 100% −
VI 100% +
100
50
CLOTTING TIME seconds
I
II
III
IV
V
VI
½
2
4
6
INCUBATION PERIOD min

TABLE I Clotting of Salt treated Mixtures on Dilution after Stated Incubation Periods Seconds 24°C

	MIXTURE + 4 5% NaCl	ADDED with water	INCUBATION PERIOD (minutes) ½	5	10	15	20
1	Thr + Fibr	-	150	85	15	5	
2	Thrombin	Fibrinogen	190	200	205	215	2
3	Fibrinogen	Thrombin	240	245	250	330	295

*Mixture (1) clotted in 18 3/4 min

TABLE II Clotting times with Varying Amounts of (T) Thrombin of (F) Fibrinogens (I II III see text) Containing Different Amounts of Serum Factor

0 2 ml F + 0 2 ml T (bovine Upjohn s at final unitage stated) seconds at 25°C

F	10	5	2 5	1 25	0 625 (T units/ml)
I	11 2	11 8	17 0	24 4	39 2
II	9 8	12 1	16 9	23 3	35 8
III	6 7	9 9	13 5	19 1	26 9

TABLE III Clotting times with Varying Amounts of Calcium of Mixtures of Thrombin and Fibrinogen Containing Different Amounts of Serum Factor

0 2 ml F + 0 1 ml $CaCl_2$ (final molarity stated) + 0 1 T (5 units/ml) seconds at 25°C

F	0 02	0 01	0 005	0 002	0 001	O(H_2O) Ca(mol)
I	18 2	10 9	9 7	9 8	10 5	12 0
II	22 2	10 8	9 6	9 7	10 3	12 6
III	15 5	10 7	9 0	5	9 8	12 6

TABLE VI Specific Assays of Eluates

Unitage per ml expressed as percentage of standard normal human plasma values (100 percent mean) PTC and AHF assays are given in Table XXXVII B = benzene extracted (see text)

	ELUATE (modified)	PROTHR (2 stage)	PROCONV (1 stage)	PROACCEL (1 stage)	THR	PTC	AHF
a	Untreated	183	100	-	0		
b	Dialyzed	167	100	trace	0	+	+
c	Dial (b e)	171	115	trace	0		
d	B e (only)	183	105	1	0		

TABLE VII Test for Antithrombin in Dialyzed Eluate and in Serum AcG Preparation both reagents benzene extracted

	INCUBATION PERIOD (minutes)	1/4	5	10	15	20	30
1	Saline control	26 1	26 9	24 6		24 0	23 9
2	Eluate	34 4	34 7	35 7		36 -	38 8
3	AcG (I)	23 3	33 0	128 4	195 0	267 0	
4	AcG†	22 0	25 8	29 9	31 6	35 1	42 8

Clotting times (sec) for 0 2 ml fibrinogen + 0 2 ml samples of thrombic mixture (T M) kept in siliconed tubes and tested (in glass) after the stated incubation periods T M 1 0 ml thrombin (bovine Upjohn s 20 units/ml) + 1 0 ml of (1) saline (2) eluate; or (3) AcG (I) untreated (4) AcG† partly purified (see Experiment 10 p 75)

TABLE IV Clotting of Platelet free (Dog) Plasma Effects of Glass (G) vs Silicone (S) Surfaces and ' Thromboplastic Agents viz (1) Platelets (A) Intact (B) Disintegrated and (2) Cephalin Clotting times seconds at 38°C after optimal recalcification

ADDED	SALINE		PLATFLETS (A)		PLATELETS (B)		CEPHALIN	
SURFACE	S	G	S	G	S	G	S	G
S	∞	∞	435	170	465	185	155	60
*S	∞	∞	550	195	660	235	150	60
*G+	∞	∞	180	170	210	205	30	30

*Held at 34°C for 1 hr with silicone (S) or powdered glass (G+)

TABLE V Clotting of Platelet free (Dog) Plasma Optimally Recalcified with Additives Noted Seconds 38°C

	ADDITIVE	CLOTTING TIME
1	Saline	∞
5	Soluplastin	7
6	Cryst trypsin (100)	118
7	Fibrinolysin (4 mg)	127

TABLE IX A Clotting Times (seconds) at 28°C for Mixtures Containing Dialyzed Eluate which had been Extracted with Benzene before Dialysis 5 Days Previously

	SAL	ELUATE	AcG b e	EXTR	CEPH 0 5"	FIBR b e	Ca 0 02M	CLOTTING TIME
1	0 1	0 1	0 1			0 4	0 3	207 6
2	0 2	0 1				0 4	0 3	340 0
3	0 1	0 1		0 1		0 4	0 3	280 6'
4	0 1	0 1			0 1	0 4	0 3	218 6
5		0 1	0 1		0 1	0 4	0 3	57 6

B *Clotting* Times of Above Eluate at Various Periods After Re extraction with Benzene

								5 15 min	3 hr
1	0 1	0 1	0 1			0 4	0 3	243 4	(443 5)
2	0 2	0 1				0 4	0 3	721 0	509 6
3	0 1	0 1		0 1		0 4	0 3	483 1	273 5
4	0 1	0 1			0 1	0 4	0 3	230 3	180 0
5		0 1	0 1		0 1	0 4	0 3	69 9	36 (6 hr)

TABLE VIII Clotting Test Mixtures (ml) of Fibrinogen (b e) and Dialyzed Eluates (see text) Effects of Benzene Extraction

Clotting times seconds at temperature stated

TEST	SAL	ELUATE	AcG (b e)	CEPH (0 5%)	FIBR (b e)	Ca (0 02 M)	CLOT TIME (26°C)
1	0 2	-	0 1		0 4	0 3	475
2	0 1		0 1	0 1	0 4	0 3	450
3	0 4	0 1 (b e)	0 1		0 4	-	2250+
4	0 3	0 1 (b e)	0 1	0 1	0 4		2340
5	0 2	0 1 (b e)	-		0 4	0 3	1005
6	0 1	0 1 (b e)	-	0 1	0 4	0 3	221
7	0 1	0 1 (b e)	0 1		0 4	0 3	210
8		0 1 (b e)	0 1	0 1	0 4	0 3	69 6
9	0 2	0 1 (untr)			0 4	0 3	354
10	0 1	0 1 (untr)		0 1	0 4	0 3	151
11	0 1	0 1 (untr)	0 1		0 4	0 3	107 5
12		0 1 (untr)	0 1	0 1	0 4	0 3	46 3

SUPPLEMENTARY TESTS substituting tissue thromboplastin* for the cephalin

TEST	SAL	ELUATE	AcG (b e)	CEPH (0 5%)	FIBR (b e)	Ca (0 02 M)	CLOT TIME (37°C)
13		0 1 (b e)	0 1	0 1*	0 4	0 3	16 7
14		0 1 (untr)	0 1	0 1*	0 4	0 3	15 7

TABLE XI Thrombin Formation from Undialyzed Eluates with Varying Activators

A Components of Thrombic Mixtures (ml)

THR MIXT	SAL	IMID BUFF	ELUATE	PRO C	AcG 1 8	CEPH 0 5"	TPLN	$CaCl_2$ 0 15M
4	3 9	0 5			0 1(b e)			0 5
5	3 8	0 5	0 1(b e)		0 1(b e)			0 5
6	3 7	0 5	0 1(b e)		0 1(b e)	0 1		0 5
7	3 8	0 5	0 1(b e)		0 1			0 5
8	3 7	0 5	0 1(b e)		0 1		0 1	0 5
9	3 6	0 5	0 1(b e)	0 1	0 1		0 1	0 5
10	3 7	0 5	0 1		0 1		0 1	0 5
11	3 6	0 5	0 1	0 1	0 1		0 1	0 5

B Clotting Times (seconds) for 0 2 ml fibrinogen + 0 2 ml thr mixt tested after stated incubation periods 28°C

TEST	MINUTES								
	1	2	3	5	7	10	30	60	90
4	1830					1346	932	750"	733
5	538					480		290	259"
6	84	43 8	34 2	21 4	18 3	21 7			
7	895"			450		216	177	181	
8	21	16 3	19	20			-		
9	23 3	18 8		20 8					
10	21 5	20 8	18 3	21 3					
11	19 8	17 6	21 6"	21 7					

TABLE X Thrombin Formation from Benzene-Extracted Dialyzed Eluate

A Components of Thrombic Mixtures (ml)

THR MIXT	IMID BUFF SALINE	ELUATE b e dial	AcG ($BaSO_4$ ads) b e (x 2) 1 8	EXTRACTIVE from plasma	$CaCl_2$ (0 1M)
1	4 6	0 1			0 3
2	4 5	0 1	-	0 1	0 3
3	4 4	0 1	0 1	0 1	0 3

B Clotting Times (sec) first visible fibrin for 0 2 ml fibrinogen + 0 2 ml Thr Mixt tested after incubation periods stated 28°C

TEST	1 min	5 min	10 min	20 min	30 min	1 hr	2 hr	3 hr	48 hr
1	1170		1230	745	720	720	710	700	745
2	676		488	503	435	395	334	335	365
3	480	347	336	259	259	243 '	249	262	560

TABLE XIII *Effects of Purified* Antihemophilic Globulin (AHG) on Thrombin Formation from Eluate

The eluate (see text) provides prothrombin proconvertin PTC (etc ?) AcG Ca and the stated THROMBO PLASTIC AGENTS are added (+) with or (0) without AHG Method III procedure at 27°C

Clotting times (seconds) for 0 2 ml fibrinogen + 0 2 ml thrombic mixture (T M) after stated incubation periods

T M	AHG	THROMBOPL AGENT	INCUBATION PERIOD (minutes)										
			1	2	3	5	7	10	15	20	30	60	1080
1	+	O	506			309		202		175*	110	100	111
2	+	Solupl 1/1	19 9	16 8	15.9	16 6			16 9				
3	O	Solupl 1/1	22 2	17 6	15.9	15 9	15 9	16 9					
4	+	Solupl 1/5	81 6	31 7	22 7	19 6	17 5	17.1	18 1				
5	O	Solupl 1/5	72 4	41 5	25 1	18	17 3	15.4	17 6				
6	+	Ceph 0 1%	233	32 3	16 8	15.8	15.7	16		16 2	18 6		
7	O	Ceph 0 1%	141	104	69 6	40 1	34	28*	25.5	26 9			

TABLE XII Reference Assays (Method III prothrombin 2 stage) of Standard (Canine) Eluate at Varying Dilutions

Thrombic Mixture (T M): 3 7 ml saline 0 5 ml imidazole buffer (pH 7 3) 0 1 ml Soluplastin 0 1 ml AcG (1 5)
0 1 ml eluate (serial dilutions) 0 5 ml $CaCl_2$ (0 15M)

Clotting times seconds for 0 2 ml std (dog) fibrinogen + 0 2 ml T M after stated incubation periods 27°C

T M	Conc	INCUBATION PERIODS (minutes at 27°C)										
	ELUATE	1	2	3	4	5	7	8	9	11	15	20
1	150%	18 3	15 1	14 6	16 7							
2	125%	19 8	15.1	15 7								
3	100%	24 5	19 7	18 5	19 9							
4	80%	26 1	20 1	20 1	19.5	19 8						
5	60%	32 1	23 3	21.5	21 6	23 1						
6	50%	39 7	26 3	23.6	24 9							
7	40%	45 5		26.9	27 1	28 1						
8	30%	64 7		33 6	32.3	37 8						
9	20%	81 4		37 5	35.7	38 5						
10	10%	200 2'		73 8		62 4	60.9	62 2				
11	5%	317 3				97 3	88.4		92 4			
12	1%	1065				447	342		200 2	180	169.7	177 5

0 15 ml and 0 125 ml of eluate with corresponding reduction of the saline were used for T M (1) and (2) respectively The 0 1 ml (undiluted) is the chosen standard = 100 percent

TABLE XIII Effects of Purified <u>Antihemophilic Globulin</u> (AHG) on Thrombin Formation *from* Eluate

The eluate (see text) provides prothrombin proconvertin PTC (etc ?) AcG Ca and the stated THROMBO PLASTIC AGENTS are added (+) with or (0) without *AHG* *Method III* procedure at 27°C

Clotting times (seconds) for 0 2 ml fibrinogen + 0 2 ml thrombic mixture (T M) after stated incubation periods

T M	AHG	THROMBOPL AGENT	INCUBATION PERIOD (minute)										
			1	2	3	5	7	10	15	20	30	60	1080
1	+	O	506			309		202		175	110	100	111
2	+	Solupl 1/1	19 9	16 8	<u>15.9</u>	16 6			16 9				
3	O	Solupl 1/1	2_ 2	17 6	<u>15.9</u>	15 9	15 9	16 9					
4	+	Solupl 1/5	81 6	31 7	22 7	19 6	17 5	<u>17.1</u>	18 1				-
5	O	Solupl 1/5	72 4	41 5	25 1	18	17 3	<u>15.4</u>	17 6				-
6	+	Ceph 0 1%	233	32 3	16 8	<u>15.8</u>	<u>15.7</u>	16		16 2	18 6		
7	O	Ceph 0 1%	141	104	69 6	40 1	34	28	<u>25.5</u>	26 9			

TABLE XIV Effects of Sequestrene Decalcification on Thrombic *Activity of Prothrombin + Activator Mixtures Tested at Different* Times from Start of Thrombin Formation

Two stage method at 28°C as described in text Clotting times (seconds) for tests at stated periods timed from start of experiment

(A) T M incubated alone and EDTA added to fibrinogen
(B) T M incubated with EDTA from 5th minute
(C) T M incubated with EDTA from 68th minute

TEST SERIES	TIME OF TEST (minutes) from start of incubation of original thrombic mixture (A)									
	2	4	5	10	15	30	60	68	78	128
A	3600+ '	609	297	86 9	78 2	85	97			
B			312	397	-	620	1440+			
C								74	73 5	73

TABLE XV Sequestrene Reversal of Thrombin Formation

Two stage method at 28°C as described in text Clotting times (seconds) *for* tests at stated periods from start of experiment (A B) or from start of incubation (C D)

MIXTURE	INCUBATION PERIOD (minutes)									
	1/4	1	2	3	5	10	30	60	90	185
(A) T M (*alone*)			80 3		44 6	47 8	46 7	45 1"	46 4	47
(B) T M + EDTA			108 4		117	135	152 4	174	195	209"
(C) Dialyzed (B)	189	184	186	181	183	192	211			
(D) T M (*alone*)		146	34 6	25 6	25 5"	26				

TABLE XIV Effects of Sequestrene Decalcification on Thrombic Activity of Prothrombin + Activator Mixtures Tested at Different Times from Start of Thrombin Formation

Two stage method at 28°C as described in text Clotting times (seconds) for tests at stated periods timed from start of experiment

(A) T M incubated alone and EDTA added to fibrinogen
(B) T M incubated with EDTA from 5th minute
(C) T M incubated with EDTA from 68th minute

TEST SERIES	TIME OF TEST (minutes) from start of incubation of original thrombic mixture (A)									
	2	4	5	10	15	30	60	68	78	128
A	3600+'	609	297'	86 9	78 2	85	97			
B			312'	397'		620	1440+			
C								74	73 5	73

TABLE XVIII Effects of Treatment with Dowex '50' Upon the Clotting Times of Various Thrombic Mixtures (see text)

Clotting times (seconds) at 28°C for 0 2 ml (or equivalent) test mixture + 0 2 ml 1% Bovine Fibrinogen after stated periods subsequent to adding agent cited

TEST SERIES	AGE OF T M	ADDED AGENT	INCUBATION PERIOD (minutes) 1	3	5	10	15	20	30	60
A		Activ Mix	+	1320	217 5	33 5	26 5	29		37
B	10 m (A)	Dowex '50'	44 9		150 6	380		496	510	
C	60 m (A)	Dowex '50'	56 2		98 1	125 3		141 8	152 3'	-
D	20 m (B)	Activ Mix	2\3		203.6	254 5		321 6	-	
*E		Activ Mix			26 '	25"	22.8	23 2	26 2	

* Original eluate treated for 1 hr with Dowex '50' (next day)

TABLE XVI Effects of Incubation with Oxalate upon the Clotting Times of Various Thrombic Mixtures (see text)

Clotting times (seconds) at 38°C for 1 ml test mixture + 1 ml fibrinogen after stated periods of incubation subsequent to dilution with agent stated

TEST	AGE of T M	ADDED AGENT	INCUBATION PERIOD (minutes)						
			1/4	5	15	30	60	90	120
A	11 m	water	15	15	15	15	15	15	20
B	12 m	oxalate	55	95	160	390	1380	2700	4500
C	4 hr	oxalate	40	40	45	40	60	50	50

TABLE XVII Effects of Incubation with Citrate upon the Clotting Times of Various Thrombic Mixtures (see text)

Clotting times (seconds) at 38°C for 1 ml test mixture + 1 ml fibrinogen after stated periods of incubation subsequent to dilution with agent stated

TEST	AGE of T M	ADDED AGENT	INCUBATION PERIOD (minutes'						
			1/4	5	15	30	60	90	120
D	9½ m	water	15	10	15	15	15	15	20
E	10 m	citrate	105	195	210	270	765	840	
F	3½ hr	citrate	215	150	140	145	145		145

TABLE XXI Rates and Yields (Minimal Clotting Times) of *Thrombin in Standard Thrombic Mixtures* (T M) with Varying Concentrations of Cephalin

Ceph conc (final) in micrograms (γ) per ml of T M Clotting times (seconds) at 28°C for 0 2 ml fibrinogen + 0 2 ml T M tested at the stated incubation periods → denotes tests not carried to completion

T M	CEPH CONC γ	INCUBATION PERIOD (minute.)									FINAL† THROMBIN
		5	10	15	20	30	35	40	45		
0	0	502	462	36.	334	315"			245"	→	trace
1	100	77 2	38 3	29.1	31 3	31 5					38"
2	50	52 2	26 3	25	22	23 3	27				58%
3	25	54	44	30	26 9	40					40"
4	12 5	67 3"	46 9	41 6	38 5	31		30		→	7
5	6 25	80 5	47 3	34 3	29 7	29.3		31 5			35"
6	3 13	117 1	77 6	43 6	37 8	3 3		31 5			31%
7	1 57	135	55 4	47 7	44	37 7			34 1		25%
8	0 78	203 7		108 8	77	43 8			38 1		19"

† computed as percentage of STANDARDS given in Fig 14 and Table XII

TABLE XIX Effect of Benzene Extraction During Conversion of Prothrombin to Thrombin in Ceph Ca AcG Activated Eluate

Clotting times (seconds) at 27°C for 0 2 ml (or equivalent) test mixture + 0 2 ml 1% bovine fibrinogen after stated periods subsequent to adding agent cited

TEST SERIES	AGE of MIXTURE	ADDED AGENT	INCUBATION PERIOD (minutes) 1	5	10	20	30	45
1		Activ mix		31	28 8	30 3	32 7	
2	(1) 5 m	Benzene extr	30 7'	47 8	79	150	169	172
3	(2) 70 m	Re act mix		174 5	185	242 4		

TABLE XX Treatment of Thrombin and Fibrinogen with (A) Decalcifying (Ion Exchange) Resin (B) Benzene

Clotting times (seconds) before and at stated periods after the treatment noted 0 2 ml Upjohn's bovine thrombin + 0 2 ml fibrinogen

Treatment	Before treatment	After treatment period of 10 min	20 min	30 min	60 min	5 hr
A	13 7	15 7			29	(12 9")*
B	10 9	13 5	14 3	11 2	-	

*Treated thrombin tested on untreated fibrinogen

TABLE XXIV

A *Assays of Clotting Factors in (Howell Type) Prothrombin and Precursor Plasmas* (Dog)

For methods see text Percentage units refer to standards (Fig 16) obtained with *normal* (100%) dog oxalated *plasma*

Recovery is computed (%) from (4) and (1) allowing *for two fold* dilution in (4)

	PREPARATION	FIBR	THR	PROTHROMBIN 2 stage u/ml	PROTHROMBIN 1 stage	PROCONV 1 stage	PROACCEL 1 stage	PTC	AHF
1	Citr Plasma	+ (9 3)	0	254	100%	96%	80%	++	++
2	Berkefeld Pl	+ (10)	0	100	56%	68%	25%	N T	N T
3	Defibr Plasma	trace	0	144	64%	48%	22%	N T	N T
4	Prothrombin	0	tr 45	37	10%	20%	25%	++	+
	Recovery			29%	20%	40%	50%	?	?

B *Antithrombin* Test on Howell Type Prothrombin(Pro)

Clotting times (seconds) for 0 2 ml fibrinogen + 0 2 ml sample of mixture of equal (1 ml) vols Pro + thrombin (bovine 20 *units/ml*) tested after stated incubation periods 28°C

INCUBATION PERIOD (minutes)	1/4	5	10	15	20	30
Test Clotting Times	17 6	18 8	17 9	18	17 3	17 1

TABLE XXII Effects of Varying Cephalin Concentrations on the Clotting Power Developed in Thrombic (i e Prothrombin + Ca) Mixtures

Clotting times (seconds) at 38°C for 1 ml fibrinogen + 0 5 ml T M tested at stated incubation periods (see text)

	FINAL DIL OF CEPH	INCUBATION PERIOD (minutes) 5	30	60	120	180	RETESTED (4 hr) 1 1000 Ceph added
1	1×10^{3}	20	7	7	7	7	12
2	1×10^{-4}	23	10''	9	9	10	12
3	1×10^{5}	90	23	18	15	15	12
4	1×10^{6}	720''	140	50'	30	30	12
5	1×10^{7}	2160	680	330	73'	65	12

TABLE XXIII Effects of Varying Thromboplastin Concentration on Activation of Prothrombin with Special Reference to Thrombin Yield

End point clotting times (seconds) at 25 ± 2°C after stated incubation periods (see text)

	FINAL CONC OF THROMBOPLASTIN	END POINT CLOTTING TIME	REQUIRED INCUBATION PERIOD
1	1 500	4	1 hr
2	1 1000	4	2 hrs
3	1 4000	6	18 hrs
4	1 40 000	8	3 days
5	1 4000 000	10	4 days

TABLE XXVI Effects of Increasing Amounts of Calcium on Thrombin Formation

Same test conditions as in Table XXV (Expt 23) except for the increased concentrations of $CaCl_2$

T.M.	Ca CONC IN T. M.	Ca CONC IN T. M. + F.	INCUBATION PERIOD (minutes) 1	5	10	20	30	60
I	8 mM	25mM	39 8	38 2	32	35 3	35 8	36 1
II	10 mM	25 mM	43 3	40 7	40	45 9	41 7	44 5
III	15 mM	25 mM	50 6	37.4	37 4	37 5	40 5	41 1
IV	20 mM	25 mM	55 3	35.9	38 1	39 3	41 6	40 8
V	25 mM	25 mM	81 9	45.4	47 3	48 2	46 1	45 5

TABLE XXVII Effects of Varying Amounts of Ca^{++} During Prothrombin Activation

T.M 5 ml total vol containing in borate buffer (pH 7 7) 1 ml 0 1% Prothrombin H (ref. [156]) + 0 25 ml 0 5% Difco's (commercial) rabbit brain thromboplastin + 0 25 ml $CaCl_2$ (final molarity cited)

Clotting times (seconds) at 29°C for 0 25 ml T M + 0 5 ml B F (0 5%) + 0 25 diluent (containing the same amount of tpln as in T M and exactly enough $CaCl_2$ to bring the final concentration of calcium to 0 0125 M in each (T M + B F.) final clotting test mixture)

T M	Ca IN T M	INCUBATION PERIOD (minutes) 1/4	5	10	15	30	60	120
1	0	127	126	125	125	129	139	145
2	0 002 M	104	12	4 5	4	4	4	4
3	0 005 M	99	6	4 5	4	4	4	4
4	0 025 M	98	6	5	4 5	4	4	4
5	0 05 M	139	75	52	21	7 5	5	4

TABLE XXV Effects of Decreasing Amounts of Calcium on Activation of Prothrombin (and Proconvertin) Containing Eluate in the Presence of Added AcG and Tissue Thromboplastin

T.M.'s: 10 ml (total) volume in imidazole buffered saline (pH 7 3) containing 0 2 ml eluate 0 1 ml AcG† '1:1) 0 5 ml ' Soluplastin and $CaCl_2$ to concentration (milli Molar) stated
Clotting tests: 0 4 ml fibrinogen + 0 2 ml (adjusted) Ca* + 0 2 ml T M at stated incubation periods
Clotting times (seconds) at 26°C

T. M.	ml 0 02 M Ca added/10 ml T.M.	Conc Ca in T.M. (mM)	Conc Ca* in T.M.+F. (mM)	INCUBATION PERIOD (minutes)						
				1	5	10	15	20	30	60
1	4 (ml)	8 (mM)	5 (mM)	47 9	43 7	42.3		44 2'	47 9	53 7
2	2 (ml)	4 (mM)	5 (mM)	58 6'	44 7	42.6		42 9	45 9	43 3'
3	1 (ml)	2 (mM)	5 (mM)	75 8	64	42.2	-		43 4	46 2'
4	0 5 (ml)	1 (mM)	5 (mM)	105 1	46 1	44 5	42.4		46 8'	46 5
5	0 25 (ml)	0 5 (mM)	5 (mM)	185 7	89 8	57 4	50 2	47 7	42 7	46 6
6	0 (buff only)	0	5 (mM)	311'	254	272		256"	319	280'

TABLE XXIX Stability of Purified Prothrombin Solution Activation by (1) Ca Alone (2) Ca + Thromboplastin (see text)

Clotting times (seconds) at 25°C of 0 5 ml fibrinogen + 0 25 T M after stated incubation periods

T M	DATE OF EXPERIMENT	ACTIVATOR ADDED	INCUBATION PERIOD									
			1 m	10 m	30 m	1 hr	2 hr	6 hr	1 d	2 d	4 d	7 d
1	Oct 8 1946	Ca (only)	112	87	66	48	38	16	9	7	3	3
2	Oct 8 1946	Ca+tpln A	68	14	6	5	3	3	3	3	3	3
3	Dec 9 1946	Ca+tpln D	20	12	8	4	3	3	3	3	3	3

TABLE XXVIII Spontaneously Formed Thrombin (II) Compared with Rapidly (Ca+tpln) Activated Prothrombin (I)

Clotting times (seconds) at 25°C for serial dilutions of the two thrombins on the same fibrinogen under the same test conditions

	RELATIVE STRENGTHS (by dilution)					
MIXTURE	100%	50%	25%	10%	5%	1%
I	4	8	11	22	40	84
II	4	8	11	22	42'	102

TABLE XXXII Thromboplastic and Anti Thromboplastic Assays on Various Lipoids and their Contaminants

Synthetic phosphatides are prepared as sodium salts
Testing methods are described in the text
Results are indicated by the following symbols
+ ++++ = varying degrees of thromboplastic (TPL IC) activity
± = slightly enhances action of standard cephalin
= varying degrees of inhibition (ANTI TP) of cephalin
N T = not tested

No.	DESCRIPTION OF PREPARATION	TPL IC	ANTI TP
1	Standard Cephalin (from dog brain) a crude mixture of phosphatides etc	++++	(>50γ/ml)
2	1 2 distearoyl α cephalin synthetic >95% pure	0	0
3	1 2 - dipalmitoyl α cephalin synthetic >95% pure	0	0
4	1 3 dimyristoyl β-cephalin synthetic >95% pure	0	0
5	stearal acetal phosphatidyl ethanolamine synthetic >95% pure	0	0
6	myristal acetal phosphatidyl ethanol amine synthetic 80 95% pure		
7	myristal acetal phosphatidyl ethanol amine repurified from 6 >99% pure	N T	0
8	palmital acetal phosphatidyl ethanol amine synthetic >99% pure (group analyses)	±	0
9	trace impurity (A_1) from preparation 6	N T	
10	trace impurity (A_2) from preparation 6	N T	
11	trace impurity (B_1) from preparation 6	N T	
12	trace impurity (B_2) from preparation 6	N T	0
13	mixture of 7 + 9	N T	
14	mixture of 7 + 11	N T	0
15	mixture of 7 + 9 + 11	N T	
16	mixture of 9 + 11	N T	
17	trace impurity (I) from preparation 8	0	-
18	trace impurity (II) from preparation 8	±	0

TABLE XXX Stability of Prothrombin and the Thrombin Formed from it by Simple Recalcification

For Details see text Clotting times (seconds) of 0 5 ml fibrinogen + 0 25 T M (dil) at stated periods
Series (A) T M (2 ml) diluted with 0 25 ml buffer before test
Series (B) T M (2 ml) maximally activated with 0 25 ml tissue thromboplastin (within 1 hr) before testing with B F End point clotting times cited 23±2°C

TEST SERIES	AGE OF T. M. BEFORE TESTING 1 hr.	1 day	2 days	3 days	8 days
A	250	85	43	25 '	4
B	4	4	4	4	4

TABLE XXXI Thrombin Formation in Presence of Various Thromboplastic Agents from Brain Tissue

Thrombic mixtures (T) = 4 cc prothrombin + 0 5 cc borate buffer + 0 25 cc N/10 $CaCl_2$ + 0 25 cc cited agents incubated for periods indicated Clotting times (seconds) * at 25°C pH - 7 75 for 0 5 cc T + 1 0 cc prothrombin free fibrinogen The P lipids are 1 1 0 00 suspensions in borate buffer

T	THROMBOPLASTIC AGENT	INCUBATION PERIOD (minutes) 10'	20'	30'	60	90'	120'	150'
0	(Calcium only)	±	±	±	±	+	+	+
1	Brain Susp	45	28	27	27	27	28	30
2	P lipid V	+	320	180	68	53	48	49
3	P lipid III	+	760	340	108	78	76	78
4	P lipid I	+	570	380	140	90	82	82
5	P lipid IV	245	120	70	38	39	40	42

* + clot starting in ½ 1 hr and becoming solid later

± weak clot starting in several hours and incomplete in 24 hours

TABLE XXXIV *Effects of Cofactor on Action of Heparin During and After Thrombin Formation*

Details of experiments are given in the text
Clotting times (seconds) at 38°C for appropriately diluted fibrinogen (see text) after incubation of T M 's for the stated periods at 7 5°C

T M	INHIBITOR	INCUBATION PERIOD (minutes) 5				90	120	TESTS ON T.M. (1) at 1 hr.	No
1		95	22	18	18	18	18	18	5
2	*Heparin*		+	900	30	23	23	19	6
3	Cofactor	40	20	19	20	25	29	28	7
4	*Hep + Cof*	∞	∞	∞	∞	∞	∞	23	8

TABLE XXXV Inhibitory *Effects of Heparin During* Prothrombin Activation by Tissue Thromboplastin

See text for details of experiment
Clotting times (seconds) at 25 ± 2°C for incubated mixtures and the fibrinogens noted in the text

T M AND TEST	INCUBATION PERIOD 5 min.	15 min.	30 min.	1 hr.	2 hr.	8 hr.	1 d.	2 d.	3 d.	7 d.
1a (without heparin)	63	42	20	9	6	4	4	4	4	4
1b (heparin in fibrinogen)	840	60	29	12	8	5	4½	4½	4½	4½
2 (with heparin)	2 hr	375	60	19	13½	10	9	8	7½	4½

TABLE XXXIII Effects of Heparin on Formation of Thrombin from Recalcified Prothrombin in Presence of Various Thromboplastic Agents

T M (see text) incubated at 15°C for periods stated Clotting times (seconds) at 38°C for 1 ml fibrinogen + 0 5 ml T M Amounts of additives per 5 ml T M

T M	THROMBOPLASTIC AGENT	INHIBITOR	INCUBATION PERIOD (minutes)					
			1	5	10	20	30	60
1	Cephalin (0 1 mg)			50	20	11	9	9
2		Heparin (0 5 mg)		∞	∞	∞	∞	∞
3	Brain P lipid (0 025 mg)		50	20	10'	8	6	6
4	'	Heparin (0 5 mg)	∞	∞	∞	∞	1800+	360+
5	Tpln Q (0 025 mg lipids)	-	35	16	9	7	7	7
6		Heparin (0 5 mg)	720	16	10	7	7	7
7	Trypsin (0 125 mg)	-	-	30	15	12	11	8
8	"	Heparin (0 5 mg)		97	32	13	11	9

TABLE XXXVII <u>PTC</u> and <u>AHF</u> Assay

Agents
0 Saline (sub trate control)
I Dialy ed eluate (p 71)
II Howell type Prothrombin (p 90)
III Purified AHG (Antihemophilic globulin (p 77))
IV Normal human plasma (<u>factor</u> control)

Methods are described in the text

The <u>ubstrates</u> (SUBS) are plasmas from cases deficient in PTC or AHF respectively

AGENT	SUBS	ORIGINAL MIXTURES			AGE OF SERUM	SERUM TESTS		PROT CONS
		(a) additive	(b) c t	(c) γ pro		(a) c t	(b) γ pro	
0	PTC	saline (control)	21 3	126	½ hr	23 9	86	32γ
0	AHF		22	115	1 hr	22 7	103	10γ
I	PTC	eluate (1 1)	22 3	112	½ hr	27 8	46	59%
I	AHF		20 9	131	1 hr	31 3	35	73γ
II	PTC	Howell Pro (1:1)	20 5	137	½ hr	29 7	40	71%
II	AHF		21 3	126	1 hr	26 5	54	57%
III	PTC	AHG (1γ)	22 4	109	½ hr	51 7	8	96γ
III	AHF	AHG (0 001γ)	21 1	129	1 hr	36 9	22	83γ
IV	PTC	Plasma (1 5)	21 9	117	½ hr	25 5	65	44½γ
IV	AHF	Plasma (1 10)	22 7	105	1 hr	63	3	97γ

TABLE XXXVI Effects of Trypsin on Activation of Prothrombin in Presence of Various Added Activators

Experimental details given in text
Percentages of prothrombin activated after stated incubation periods 25°C
T M 's: 5 ml total vol containing 4 ml Pro D (0 2%) + stated additives

T M	ACTIVATORS ADDED (ml)			INCUBATION PERIOD								
	$CaCl_2$ 0 1 M	tpln C 0 1%	trypsin 40 u/ml	5 min	15 min	30 min	1 hr	3 hr	6 hr	24 hr	30 hr	48 hr
I	0 25				±	±	+	1%	4%	40%	80%	100%
*II			0 5	-	+	1%	1%	8%	20%	2%	+	
*III	0 25		0 5	6%	25%	35%	60%	100%	80%	4%	1%	
IV	0 25	0 75		40%	80%	100%	100%	100%				
V	0 25	0 25		1%	5%	9%	20%	30%	35%	70%	80%	100%
*VI	0 25	0 25	0 5	35%	42%	60%	75%	100%	50%	3%	1%	

*NOTE, Clot lysis occurred in 3 days in tubes containing trypsin

TABLE XL Effects of (A) Weak Thrombin and (B) Fibrinolysin upon the Thromboplastic Action of Platelets

Experimental details given in text

T.M.: 0 2 ml Pro 0 1" + 0 2 ml AcG 0 1" + 0 5 ml $CaCl_2$ (0 05 M) the calcium being added after the other activators cited making with the borate buffer (pH 7 7) diluent a total 5 ml vol

Clotting times (seconds) at 26°C for 0 5 B F (1%) + 0 25 T M at the stated incubation periods

T M	ACTIVATORS (ml/5 ml T.M.)	INCUBATION PERIOD (minutes) 5	10	20	40	60
0	0 (control)	885	913	920	970	1010
1	Platelets (0 2)	810	620	330	190	97
2 (A)	Plat (0 2) + Thr *(0 5)	145	52	22	12	9
3 (B)	Plat (0 2) + Lysin (0 6)	60	46	15	12	9

* Special thrombin see text

TABLE XXXVIII Potentiation of Thromboplastic Action of Platelets by (A) Antihemophilic Globulin and (B) Trypsin

Experimental details given in text All T M 's contain 0 1 ml dialyzed eluate 0 1 ml AcG (1:5) and 0 5 ml 0 02M $CaCl_2$ with imidazole buffered saline to 5 ml total volume

Clotting times (seconds) at 28°C *for 0 2 ml fibrinogen + 0 2 ml T M* after stated incubation periods

T M	(ml) OTHER ADDITIVES	INCUBATION PERIOD (minutes) 1	5	10	20	30	60
(A) 1	(0 2) platelets	2 hr	495 2	65	31 9"	30.3	32 3
2	(0 2) platelets (0 2) AHG (1%)	2 hr	92 6	44 9	42 7	40.1	45
3	(0 2) AHG (1%)		1 hr	582 5	349 6	164.2	246 1
(B) 4	(0 2) platelets (0 1) trypsin	155 5	42 1	35.7'	39 8	37 5	38 8
5	(0 1) trypsin	-	2 hr	1609+	791	570	445
6	(0 1) trypsin (0 2) AHG (1%)		732 6	242 2	195 4	172.6	174 2

TABLE XXXIX Potentiation of Thromboplastic Action of Platelets by (C) Weak Thrombin and (D) Fibrinolysin

Experimental details given in text All T M 's contain 0 1 ml dialyzed eluate 0 1 ml AcG and 0 5 ml 0 02M $CaCl_2$ with imidazole buffered saline to 5 ml total volume

Clotting times (seconds) at 26°C for 0 2 ml fibrinogen + 0 2 ml T M after stated incubation periods

T M	(ml) OTHER ADDITIVES	INCUBATION PERIOD (minutes) 5	10	20	30	60	90
(C) 7	(0 2) platelets (0 2) thrombin	191	53 5	29 5	30 3	26 9	27 7
(D) 8	(0 2) plate ets (0 1) lysin	194	66 6	39	34 4	31 3	29 5
	^ on (control)	2 hr	⌐ hr	2 hı	1560	820	

TABLE XLII Eff ts of Anti P ot n on Th ombopl tl Acti f Pl t l t Fib inoly i Additi to Th ombi Fo ming Sy t ms

T.M.s in T bl XLI ept f th b tituti not d in th t xt

Clotting times (nd) at 25 C in the l t t

T M	ACTIVATORS	INHIBITORS	INCUBATION PERIOD (minutes)				
			5	10	20	40	60
6	fib inoly i	0	390	380	368	335	320
7	pl t l t + ly in	0	29 1	23	22	22	22
8	pl t l t + ly in	P I	28	22 5	19 9	20	20
9	pl l t + ly in	Afln	37 6	24 9	22 4	22 2	20
10	pl t l t + ly i	S B I	630	135	42	38 5	35 4
11	0 (C A G nly)	0	∞	∞	∞	∞	∞

∞: N l t in ½ h
P t b i 15 ml

TABLE XLIII Eff t f An i P ot th A tl f Pl t l (I) Without AHG (II) With AHG (Antih m phili Gl bulin) in Th mbl F mi g Sy m

T.M. s 5 ml in bo b ff (pH 7 7) t l 0 2 ml l t (di ly d) 0 2 ml pl t l t + 0 2 ml l s AHG (in II) 0 1 ml A G (1 8) 0 1 ml 0 1 inhibi (0 3 ml f 0 4% i of th a ti fib in ly in t 5) + 0 5 ml C Cl (0 04 M)
I t t 4 5 and 9 th pe ti inhibit w p i b t d with th ti t (I ID f 15 min t t 28 C bef ddi g th th m p f th T M

Clotting times (ond) t 28 C f 0 2 ml fib in g 0 2 ml T M t th d in b i pe iod

T M	AHG (1 s)	INHIB	INCUBATION PERIOD (minutes)					
			1	3	5	10	20	30
(I) 1	0	0	319		69 8	37 8	29 9	29 9
2	0	S B I			951	165	88 5	77 4
3	0	P I		62 7	36 7	28	24 5	26 9
4	0	N B I			36 2	26	25 3	23 4
5	0	Afl			833	534	410	292
(II) 6	0 2 ml	0	67		39 6	35 3	36 1	39 5
7	0 2 ml	S B I			621	316	264	251
8	0 2 ml	P I		34	31 5	37 2	39	37 4
9	0 2 ml	N B I*			35 3	32 2	24 7	34 8

TABLE XLI Effects of Anti Proteases on Thromboplastic Action of Platelet Trypsin Additions to Thrombin Forming Systems

T.M.'s 5 ml in borate buffer (pH 7 7) containing 0 2 ml Pro (0 1%) + 0 2 ml AcG (0 1%) + 0 1 ml $CaCl_2$ (0 05 M) added after the various other activators and inhibitors cited The asterisk (*) with the inhibitor denotes a 15 minute (room temperature) pre incubation witr the activator system

Clotting times (seconds) at 25°C for 0 5 ml B F (1%) + 0 25 ml T M at successive incubation periods Clot-lysis followed over 3 weeks

T M	ACTIVATORS	INHIBITORS	INCUBATION PERIOD (minutes)					CLOT LYSIS
			5	10	20	40	60	
1	platelets	0	720	540	270	100	65	0
2	trypsin	0	184	140	95	58	51	3 d
3	platelets + trypsin	0	47	29 5	26 5	23 5	23 2	0
4	platelets + trypsin	P I	660	240	175	113	96	0
5	platelets + trypsin	Afin	[illegible]	[illegible]	205	103	71	0

TABLE XLVI Lack of Effects of Anti Proteases on the Thrombin Fibrinogen Reaction

Clotting times (seconds) at 25°C for 0 5 ml fibrinogen + 0 25 ml thrombin (5 units/ml) + 0 25 anti protease (or buffer in control) the last two* being pre incubated with thrombin for the periods noted

	ADDITIVE	PRE INCUBATION PERIOD (minutes)				
		¼	10	20	30	60
0	Buffer	14 8	15	15	14 6	15 3
1	P I	17	18 5	18 5	19 5	20 5
2	S B I	14 2	16	17	17 1	17
3	N B I *	15 2	16	16 5	16 7	16 8
4	Afin *	15	16	16	15 5	16 4

TABLE XLVII Proteolytic Activities of <u>Old</u> Enzyme Preparations and Their Inhibition by Anti Proteases

Experimental details and descriptions of the enzyme preparations are given in the text

The pancreatic inhibitor (P I) acts immediately whereas the navybean inhibitor (N B I *) and antifibrinolysin (Afin *) require 15 20 minute pre incubation with the enzyme before adding the fibrinogen to make the L M

<u>Clotting times</u> (seconds) at 28°C for 0 2 ml thrombin (20 units/ml) + 0 2 ml L M after incubation periods stated

L M	ADDITIVES	INCUBATION PERIOD (minutes)		
		10	20	30
(A) 1	0 (buffer)	14 2	14	14 3
2	trypsin	540	840	∞
3	trypsin + N B I *	14	15	14
4	trypsin + P I	15 7	22	28
(B) 5	fibrinolysin	∞	∞	∞
6	lysin + N B I *	21	22	18
7	lysin + Afin *	13	13 8	14 8

TABLE XLIV Effects of Anti-Proteases on the Action of Tissue Thromboplastin in Thrombin Forming Systems

T.M.'s 5 ml in borate buffer (pH 7 7) contain 0 2 ml Pro (0 1%) + 0 5 ml tpln A + 0 2 ml AcG (0 1%) + 0 5 ml $CaCl_2$ (0 05M) added after the various inhibitors cited In (4) the thromboplastin was pre incubated for 15 minutes at 25°C with the anti-fibrinolysin *

Clotting times (seconds) at 25°C for 0 5 ml B F (1%) + 0 25 ml T M at successive incubation periods

T M	INHIBITOR ml (conc.)	INCUBATION PERIOD (minutes) 5	10	20	40	60
1	0	22 5	17 1	15 2	15	14 2
2	S B I 0 2 (0 01%)	450	420"	450'	450	380
3	P I 0 2 (0 1%)	28 8	23 3	21 8	21 5'	20
4	Afln * 0 3 (0 5%)	28 5	22 4	20 6'	21 2	21"

TABLE XLV Effects of Anti Proteases on the Action of Cephalin in Thrombin Forming Systems

T.M.'s 5 ml in borate buffer (pH 7 7) contain 0 2 ml eluate (dialyzed) + 0 1 ml cephalin (0 1%) + 0 2 ml AcG (1:8) + 0 5 ml $CaCl_2$ (0 04 M) added after inhibitors stated In test (4) the cephalin was pre incubated for 15 minutes at 28°C with the N B I *

Clotting-times (seconds) at 28°C for 0 2 ml (dog) fibrinogen + 0 2 ml T M at successive incubation periods

T M	INHIBITORS ml (conc %)	INCUBATION PERIOD (minutes) 5	10	20	30
1	0	56 5	52	50 8	56 4
2	S B I 1 0 (0 001)	222	156 5	149 1	163
3	P I 0 1 (0 1)	48 8	50 3	57	59 8
4	N B I 0 1 (0 1)	46 2	44 2	49 4'	53 6

TABLE XLIX Effects of Heparin on Thrombin Forming Mixtures Activated by (I) Platelets; (II) Platelets + Trypsin; or (III) Tissue Thromboplastin (See text)

T.M.'s: 5 ml in imidazole buffered saline (pH 7 3) *containing 0 2 ml* eluate (dialyzed) + 0 1 ml AcG (1:8) + 0 ₋ ml (I) platelets or (II) platelets + 0 5 ml trypsin (0 02") or (III) Soluplastin (0 2 ml) + 0 5 ml (a) buffered saline or (b) heparin (2 units/ml) + 0 5 ml $CaCl_2$ (0 04 M)

Clotting times (seconds) at 28°C for 0 ₋ ml fibrinogen + 0 1 ml *(c) heparin (0 2 units/ml)* or *(d)* buffered saline + 0 1 ml T M after stated incubation periods

T M	ACTIVATORS	HEPARIN in T. M.	INCUBATION PERIOD (minutes)								
			3	5	8	10	15	20	25	30	35
I 1 (a)	(d) Platelets	0		64		29 9		29 2		29 5	
1 (a)	(c) Platelets	0	98 6		24 1		23 8		23 6		24
2 (b)	(d) Platelets	+								1000+	
II 3 (a)	(c) Plat + tryp	0	61 4	40 6		36 4				35 8	34
4 (b)	(d) Plat + tryp	+	172 5	64 5		47 6		35 4		37 2	
5 (a)	(d) Trypsin	0	570	495		392	352	407		420	
III 6 (b)	(d) Soluplastin	+	37 1	35 8		31 7				30 1	

TABLE XLVIII Effect of Washed Platelets as an Accelerator of the Activation of AcG Poor Prothrombin

Reagents and experimental details are described in the text

T.M. 5 ml in borate buffer (pH 7 75) containing 0 2 Pro (0 1%) + 0 5 ml $CaCl_2$ (0 05 M) + the various activators noted

Clotting times (seconds) at 26°C of 0 5 ml B F (1%) + 0 25 ml T M at successive incubation periods

T M	ACTIVATORS ml (dilution)	INCUBATION PERIOD (minutes) 5	10	20	40	60
1	Tpln B 0 5	420	300	202	141	110
2	Tpln 0 5 + Plat 0 2	390	240	80	22 4	15
3	Tpln 0 5 + AcG 0 1 (0 001%)	300	140	52	25 8	20 3
4	Tpln 0 5 + AcG 0 1 (0 1%)	11 4	9 4	8 0	7 8	7 6

TABLE XLIX *Effects of Heparin on Thrombin Forming Mixtures Activated by (I) Platelets; (II) Platelets + Trypsin or (III) Tissue Thromboplastin* (See text)

T.M.'s: 5 ml in imidazole buffered saline (pH 7 3) containing 0 2 ml eluate (dialyzed) + 0 1 ml AcG (1:8) + 0 2 ml (I) platelets or (II) platelets + 0 5 ml trypsin (0 02%) or (III) Soluplastin (0 2 ml) + 0 5 ml (a) buffered saline or (b) heparin (2 units/ml) + 0 5 ml $CaCl_2$ (0 04 M)

Clotting times (seconds) at 28°C for 0 2 ml fibrinogen + 0 1 ml (c) heparin (0 2 units/ml) or (d) buffered saline + 0 1 ml T M after stated incubation periods

T M	ACTIVATORS	HEPARIN in T. M.	INCUBATION PERIOD (minutes) 3	5	8	10	15	20	45	30	35
I 1 (a)	(d) Platelets	0		64		29 9		29 2		29 5	-
1 (a)	(c) Platelets	0	98 6		24 1		23 8		23 6		24
2 (b)	(d) Platelets	+						-		1000+	-
II 3 (a)	(c) Plat + tryp	0	61 4	40 6		36 4				35 8	34
4 (b)	(d) Plat + tryp	+	172 5	64 5		47 6		35 4		37 2	
5 (a)	(d) Trypsin	0	570	495		392	352	407		420	
III 6 (b)	(d) Soluplastin	+	37 1	35 8		31 7				30 1	

TABLE L Effects of Platelets on the Thrombin Fibrinogen Reaction

Method: 0 2 ml thrombin (conc cited) + (1) 0 1 ml platelets or (2) 0 1 ml saline + 0 2 ml fibrinogen

Clotting-times (seconds) at 26°C

ADDED THR (units/ml):	20	10	5	2 5	1 25
(1) with platelets	10 5"	17 1"	31 5"	59 0'	129 (130)
(2) control with saline	10 5	17 8	31 0	59 5'	105" (105 8)

TABLE LI Platelet Utilization During Clotting of (A) Normal and (B) Hemophilic Blood

Platelet counts (x1000/mm^3) after stated times in siliconed tubes at 37°C (I) in clotting bloods (II) in sequestrene plasma ('aging' control)

TIME (minutes)	(A) NORMAL BLOOD (I)	(B) HEMOPHILIC BLOOD (I)	(II) CONTROL
0	320	182	
1½	290	154	
2	265	192	
3	196	132	
4	238	150	
5	103	125	
6	106 (clotting)		
7	34	137	
8	31	-	
9	19	-	
10	31	143	
12 5	15	136	
15	18	160 (clotting)	
30	3 3	51	
45	4 2	14	
60	3 7	2 4	(A) 252
75		2	
90	-	1 7	
120		4	(B) 174

TABLE LII GROUP I (A) Normal adults; (B) Healthy parturient mothers; (C) Normal newborn infants (cord bloods)

1	No (Sub group)	>50 (A)	32 (B)	32 (C)
2	Cases	Normal Adults	Mothers	Newborns
3	Age	19 55	15 43	0
	Sex	F M	F	F M
	Race	W C	23W 9C	23W 9C
4	Date(s) Exam	1950 55	1953 55	1953 55
5	Duration Bleeding	neg	neg	neg
6	Fam Hist of Bleeding	neg	neg	neg
7	Bleed Tendency	neg	neg	neg
8	Tourniquet Test	neg	N T	N T
9	Bleed time (min)	6 (2 10)		
	Clotting Time:			
10	glass (min)	9 (5 13)	7 (2 12)	5 (1 10)
11	silicone (min)	33 (15 50)	24 (6 60)	12 (3 33)
12	Clot Retraction	+++(+)	+++(+)	++++ +
13	Fibrinolysis	neg	neg	neg (+ 2 cases)
14	Platelet Count x 1000/mm³	249 (143 356)	241 (105 446)	271 (136 523)
15	Platelet Tpln *	100 (65 150)	80 100+	80 100+
16	Platelet Accel *	100 (40 150)	60 100+	50 100+ (one 10)
17	Serum Serotonin*	100 (50 150)	N T	N T
18	Platelet Agglut	neg	N T	N T
19	Plasma Thr C T	15 (13 17)	16 5 (av)	25 5 (av)
20	Serum Antithrombin	variable	normal	normal
21	Fibr mg/100 ml	364 (200 540)	652 (340 1020)	344 (192 800)
22	AHF*	100 (60 135)	60 100+	60 100+
23	PTC*	100 (60 135)	110 (83 143)	59 (15 90)
24	Inhibitors etc	neg	neg	neg
25	Prothr Cons %	95 (90 99)	93 (80 96)	83 (55 95)
26	Prothr Time*	100 (74 133)	119 (68 133)	119 (56 133)
27	Prothrombin*	100 (70 136)	115 (81 150)	43 (30 59)
28	Proconvertin*	100 (64 138)	135 (100 216)	51 (22 80)
29	Proaccelerin*	100 (60 150)	70 100+	100 300
30	Remarks	Med School personnel	Parity 1 11	125 others excluded

*percent of standard normal used throughout Tables LII LVIII

TABLE LIII A Group II Congenital (Familial) Thrombocytopenias

1	No	Case 1		Case 2
2	CASE	A B		D D
3	Age	2	3	9
	Sex	M	M	M
	Race	C	C	C
4	Date(s) Exam	1 Feb '54	20 Apr '55	20 Apr '55
5	Duration Bleeding	1 16 mo	-	-
6	Fam Hist of Bleeding	+	+	+
7	Bleeding Tendency	+++	0	0
8	Tourniquet Test	++++	neg	+(+)
9	Bleeding time (min)	60	2½	6½
	Clotting Time			
10	glass (min)	6½	6½	5 3/4
11	silicone (min)	6½	7½	29½
12	Clot Retraction	0 ±	+++	++
13	Fibrinolysis	0	0	0
14	Platelet Count x 1000/mm^3	38	320	98
15	Platelet Tpln *	N T	50	20
16	Platelet Accel *	N T	80	10
17	Serum Serotonin*	N T	N T	N T
18	Platelet Agglut	N T	N T	N T
19	Plasma Thr C T	±	±	±
20	Serum Antithrombin	+	N T	N T
21	Fibr mg/100 ml	320	N T	520
22	AHF*	100	100	100
23	PTC*	80	80	70
24	Inhibitors etc	N T	N T	N T
25	Prothrombin Cons %	95	100	95
26	Prothrombin Time*	125	93	88
27	Prothrombin*	111	106	93
28	Proconvertin	95	100	110
29	Proaccelerin*	100+	100+	100
30	Remarks	*Before treatment*	*After splenect*	

*percent of standard normal

TABLE LIII B Group III Hereditary Thrombasthenia (3) and Pseudo hemophilia (4 5)

1	No	Case 3	Case 4	Case 5
2	CASE	(N P)	(A C)	(S C)
3	Age	16	52	20
	Sex	F	F	F
	Race	W	W	W
4	Date(s) Exam	9 June '53	23 Apr '55	23 Apr '55
5	Duration Bleeding	birth	33 yrs	?
6	Fam Hist of Bleeding	+	+	+
7	Bleeding Tendency	+++	+(+)	+(+)
8	Tourniquet Test	++++	+	++
9	Bleeding time (min)	60	1	6½
	Clotting Time			
10	glass (min)	9	6 3/4	9½
11	silicone (min)	22	31	N T
12	Clot Retraction	0	++++	+++
13	Fibrinolysis	neg	neg	neg
14	Platelet Count x 1000/mm^3	230	238	104
15	Platelet Tpln *	100	50	25
16	Platelet Accel *	N T	50	20
17	Serum Serotonin*	100	N T	N T
18	Platelet Agglut	N T	N T	N T
19	Plasma Thr C T	norm	N T	N T
20	Serum Antithrombin	+	N T	N T
21	Fibr mg/100 ml	520	720	700
22	AHF*	100	100	100
23	PTC*	85	90	60
24	Inhibitors etc	neg	neg	neg
25	Prothrombin cons "	95	>95	>95
26	Prothrombin Time*	94	85	71
27	Prothrombin*	89	90	100
28	Proconvertin*	111	N T	100
29	Proaccelerin*	62	N T	90
30	Remarks	Glanzmann's Thrombasthenia	Pseudohemophilia	

*percent of standard normal

TABLE LIII A Group II Congenital (Familial) Thrombocytopenias

1	No	Case 1		Case 2
2	CASE	A B		D D
3	Age	2	3	9
	Sex	M	M	M
	Race	C	C	C
4	Date(s) Exam	1 Feb '54	20 Apr '55	20 Apr '55
5	Duration Bleeding	1 16 mo	-	-
6	Fam Hist of Bleeding	+	+	+
7	Bleeding Tendency	+++	0	0
8	Tourniquet Test	++++	neg	+(+)
9	Bleeding time (min)	60	2½	6½
	Clotting Time			
10	glass (min)	6½	6½	5 3/4
11	silicone (min)	6½	7½	29½
12	Clot Retraction	0 ±	+++	++
13	Fibrinolysis	0	0	0
14	Platelet Count x 1000/mm³	38	320	98
15	Platelet Tpln *	N T	50	20
16	Platelet Accel *	N T	80	10
17	Serum Serotonin*	N T	N T	N T
18	Platelet Agglut	N T	N T	N T
19	Plasma Thr C T	±	±	±
20	Serum Antithrombin	+	N T	N T
21	Fibr mg/100 ml	320	N T	520
22	AHF*	100	100	100
23	PTC*	80	80	70
24	Inhibitors etc	N T	N T	N T
25	Prothrombin Cons *	95	100	95
26	Prothrombin Time*	125	93	88
27	Prothrombin*	111	106	93
28	Proconvertin	95	100	110
29	Proaccelerin*	100+	100+	100
30	Remarks	Before treatment	After splenect	-

*percent of standard normal

TABLE LIII B Group III Hereditary Thrombasthenia (3) and Pseudo hemophilia (4 5)

1	No	Case 3	Case 4	Case 5
2	CASE	(N P)	(A C)	(S C)
3	Age	16	52	20
	Sex	F	F	F
	Race	W	W	W
4	Date(s) Exam	9 June '53	23 Apr 55	23 Apr '55
5	Duration Bleeding	birth	33 yrs	?
6	Fam Hist of Bleeding	+	+	+
7	Bleeding Tendency	+++	+(+)	+(+)
8	Tourniquet Test	++++	+	++
9	Bleeding time (min)	60	1	6½
	Clotting Time			
10	glass (min)	9	6 3/4	9½
11	silicone (min)	22	31	N T
12	Clot Retraction	0	++++	+++
13	Fibrinolysis	neg	neg	neg
14	Platelet Count x 1000/mm³	230	238	104
15	Platelet Tpln *	100	50	25
16	Platelet Accel *	N T	50	20
17	Serum Serotonin*	100	N T	N T
18	Platelet Agglut	N T	N T	N T
19	Plasma Thr C T	norm	N T	N T
20	Serum Antithrombin	+	N T	N T
21	Fibr mg/100 ml	520	720	700
22	AHF*	100	100	100
23	PTC*	85	90	60
24	Inhibitors etc	neg	neg	neg
25	Prothrombin cons *	95	>95	>95
26	Prothrombin Time*	94	85	71
27	Prothrombin*	89	90	100
28	Proconvertin*	111	N T	100
29	Proaccelerin*	62	N T	90
30	Remarks	Glanzmann's Thrombasthenia	Pseudohemophilia	

*percent of standard normal

TABLE LIV Group IV Summary of Data on 25 Thrombocytopenics (Acquired)

		FINDINGS				REMARKS
		Pos.	Neg.	?	N.T.	
1	Nos 6 30					Diag
3	Ages 17 mo 78 yr					v text
	Sex 11 F 14 M					
	Race 22 W 3 C					
5	Duration Bleed 1 d 8 yr	19	1		5	
7	Bleeding Tend +++(+)	10				
	++	7				
	+	2				
	0	-	1	1	4	
8	Tourn Test ++++ +	19	3	2†	1	†Negroes
9	Bleed Time >10 min	17	7	1†		† 9 min
	Clotting Time					
10	glass >13 min	1	21	3†		†12½ 10½'
11	silicone >50 min	10	10	3†	2	†48 47'
12	Clot Retr (glass) ++ 0	17	3		5	
14	Platelet Count					
	x 1000/mm^3 <100 000	24		1†		†174 000
15	Platelet Tpln * <10	18	1	1†	5	†30
16	Platelet Accel * <10	19			6	
17	Serum Serotonin* <50	9	3		13	
19	Plasma Thr C T >2x norm	15		8†	2	† slight
21	Fibr (a) <200 (decr)	0	14		2	
	(b) >540 (incr)	9				
22	AHF* <60%		22	1†	2	†27 in ♀
23	PTC* <60%		21	2†	2	†60 65
25	Prothrombin Cons <90%	21	3		1	
26	Prothrombin Time* <65		24	1†		†61
27	Prothrombin* <65	3†	21		1	†52 62
28	Proconvertin* <65	1†	23		1	†55
29	Proaccelerin* <60	3†	22			†30 50

*percent of standard normal

TABLE LV Group V: Thrombocytosis

1	No	31	32	33
2	Cases	R G	J L H	E M
3	Age	63	64	23
	Sex	M	M	M
	Race	C	W	W
4	Date(s) Exam	23 June '55 (27 Aug '56)	30 Mar 54 (8 Apr '55)	15 Dec 54
5	Duration Bleeding	2 weeks	?	?
6	Fam His of Bleed	neg	neg	neg
7	Bleeding Tend	++	++	0
8	Tourn Test	neg	neg	neg
9	Bleeding Time (min)	1½ 4	3½	2
	Clotting Time			
10	glass (min)	11½	7½	4½
11	silicone (min)	30	48	46
12	Clot Retraction	++++	++++	++++
13	Fibrinolysis	neg	neg	neg
14	Platelet count x 1000/mm³	2000	1886 (550)	850
15	Platelet Tpln *	100	40	100
16	Platelet Accel *	90 (20)	50	100
17	Serum Serotonin*	N T	5	N T
19	Plasma Thr C T	normal	normal	normal
20	Serum Antithr	normal	normal	normal
21	Fibr mg/100 ml	200	540	660
22	AHF*	100	100	100
23	PTC*	90	100	80
25	Prothr Cons %	90	99	90
26	Prothr Time*	85	44 (68)	52
27	Prothrombin	88	68 (48)	60
28	Proconvertin*	100	60 (44)	78
29	Proaccelerin*	60	50 (40)	90
30	Remarks	Carcinoma of stomach general metastases bone marrow hyper plasia incr megakaryocytes	Polycythemia vera treated with P^{32} gout	Chronic granulo cytic (Myeloid) leukemia

* percent of tandard normal

TABLE LVI A Group VI (A): Leukemics with Thrombocytopenia (22 out of 39 cases studied)

1	Nos 34 55	FINDINGS				Remarks
		Pos	Neg	?	N T	
3	Ages: 13 83 yr					
	Sex: 7 F; 15 M					
	Race 19 W; 3 C					
5	Duration Bleeding:					
	1 wk 3 mo					
7	Bleeding Tend ++++	2				
	' +++	0				
	++	5				
	' ' +	5				
	' 0		6		4	
8	Tourn Test ++++ 0	5	16		1	
9	Bleed Time: >10 min	7	15			
	Clotting Time:					
10	glass: >13 min		20	2 †		†12' (2 cases)
11	silicone: >50 min	15	6	1†		†45'
12	Clot Retr : ++	16	2	4 †		† clots lysed (see 13)
13	Fibrinolysis	4	18	-		
14	Platelet Count <100 000 x 1000/mm³	22				
15	Platelet Tpln *: <60	21		-		
16	Platelet Accel *: <40	19	1	1†	1	†50
17	Serum Serotonin* <50	5	1	-	16	
18	Platelet Agglut	5	6		11	
19	Plasma Thr C T : >2x norm	6	6	10†		†1 5 2X norm
20	Serum Antithrombin incr	6	13	3†	-	†'serum' clot
21	Fibr (a) <200 mg	1	12	1†	1	†230 mg/100 ml
	(b) >540 mg	7†			-	†560 1180 mg
22	AHF* <60		2	-	20	
23	PTC* <60	1	19	1†	1	†60
25	Prothrombin Cons <90%	16	5	1†	-	†90%
26	Prothrombin Time* <65	7	13	2†	-	†64; 65
27	Prothrombin* <70	2	20	-	-	
28	Proconvertin* < 65	3	18	1†		†66
29	Proaccelerin* <60	12	9	1†		†60

* per cent of standard normal

TABLE LVI B Group VI (B): Leukemics with Abnormal Platelet Function Tests but no Thrombocytopenia (12 out of 39 cases studied)

1 Nos 56 67	FINDINGS				Remarks
2 Cases	Pos	Neg	?	N T	
3 Ages: 6 58 yrs Sex: 5F; 7M Race: 11W; 1C					
5 Duration Bleed : (?)				12	
7 Bleeding Tend : ++ +	5	6		1	
8 Tourn Test: ++ +	3	9			
9 Bleed Time: >10 min	1	11			
Clotting Time:					
10 glass >13 min		10	2†		†12 min
11 silicone: >50 min	9	2		1	
12 Clot Retr ++ 0	3	9			
14 Platelet Count x 1000/mm³: <100 000		12†			†118 000 445 000/min ³
15 Platelet Tpln *: <60	7	4	1†		†70
16 Platelet Accel *: <40	9	1	2†		†50
17 Serum Serotonin*: <50		-	2†	10	†44 50
18 Platelet Agglut :		2		10	
19 Plasma Thr C T : >2x norm	2	9	1†		†*nearly x 2 norm*
20 Serum Antithrombin incr	2	10			
21 Fibr : (a) <200 mg	0	9			
(b) >540 mg	3†				†580 1120 mg/ 100 ml
22 AHF*: <60		2		10	
23 PTC*: <60	1†	11			†45
25 Prothrombin Cons : <90%	4	6	2†		†89 90
26 Prothrombin Time* <75	4	6	1†	1	†74
27 Prothrombin*: <70	2	10			
28 Proconvertin*: < 65	1	11			
29 Proaccelerin*: < 60	3	9			

* percent of standard normal

TABLE LVII A Group VII (A) Platelet Problems in Uremics (10 out of 11 cases studied)

		FINDINGS				
1	Nos 68 77					
2	Cases	Pos	Neg	?	N T	REMARKS
3	Ages 10 59 yrs					
	Sex: 8F 3M					
	Race: 8W; 3C					
7	Bleeding Tendency: ++++	1				
	++	2				
	+ ±	5				
	0		1	-	1	
8	Tourniquet Test	1	9		-	
9	Bleeding Time: >10 min	1	9	-	-	
	Clotting Time					
10	glass >13 min		9	1†	-	†10 min
11	silicone >50 min	2	8			
12	Clot Retraction: ++ 0		10			
14	Platelet Count x 1000/mm^3 <100 (000)		10	-		†147 574
15	Platelet Tpln * <60	7	2		1	
16	Platelet Accel *: <40	7	1	1†		†50
17	Serum Serotonin* 50 or less	2†		-	8	†16 50
19	Plasma Thr C T : (incr)	6	3		1	
20	Serum Antithrombin (incr)	1 (sl.)	7	2†	-	†decr (?)
21	Fibr (a) decr (<200 mg)	1	4			†120 mg
	(b) incr (>540 mg)	5†				
22	AHF*: <60		10	-		
23	PTC* <60		6		4	
25	Prothrombin Cons <90%	4	1	1†	4	†90%
26	Prothrombin Time* <70	2	8			
27	Prothrombin* <70	3	7			
28	Proconvertin*: <65	4	5	1†		†66
29	Proaccelerin* <60	1	9			
	N P N (or B U N) mg/100 ml	10	0			†78 330

* percent of standard normal

TABLE LVII B Group VII (B): Platelet Problems in <u>Uremics</u> (1 out of 11 cases studied)

1	No 78		
2	Case	M Tu	
3	Age	13	
	Sex	F	
	Race	W	
4	Dates Examined	31 Jan '55	30 May 55
7	Bleeding Tendency: ++	++	+++
8	Tourniquet Test	neg	neg
9	Bleeding Time (minutes)	3	3½
	Clotting Time:		
10	glass (minutes)	8	N T
11	silicone (minutes)	43	N T
12	Clot Retraction	++++	N T
14	Platelet Count x 100/mm^3	346	340
15	Platelet Thromboplastin*	100	100
16	Platelet Accel * (?)	50	100
17	Serum Serotonin*	N T	N T
19	Plasma Thr Clotting Time	norm	N T
20	Serum Antithrombin	norm	N T
21	Fibr : (a) decr (<200 mg)		
	(b) incr (>540 mg)	800	N T
22	AHF*	100	N T
23	PTC*	80	50
25	Prothrombin Cons ¶	95	N T
26	Prothrombin Time*	88	4
27	Prothrombin*	84	20
28	Proconvertin*	100	15
29	Proaccelerin*	100	22
30	Remarks	Died 31 May '55 from pyelonephritis (chr) in polycystic kidneys.	

* percent of standard normal

TABLE LVII A Group VII (A) Platelet Problems in <u>Uremics</u> (10 out of 11 cases studied)

		FINDINGS				
1	Nos 68 77					
2	Cases	Pos	Neg	?	N T	REMARKS
3	Ages: 10 59 yrs					
	Sex 8F; 3M					
	Race: 8W; 3C					
7	Bleeding Tendency: ++++	1				
	++	2				
	+ ±	5				
	0		1		1	
8	Tourniquet Test	1	9	-		
9	*Bleeding Time: >10 min*	1	9	-		
	Clotting Time:					
10	glass: >13 min		9	1†		†10 min
11	silicone: >50 min	2	8			
12	Clot Retraction: ++ 0		10			
14	Platelet Count					
	x 1000/mm³ <100 (000)		10			†147 574
15	Platelet Tpln * <60	7	2	-	1	
16	Platelet Accel *: <40	7	1	1†	-	†50
17	Serum Serotonin*: 50 or less	2†			8	†16 50
19	Plasma Thr C T : (incr)	6	3	-	1	
20	Serum Antithrombin (incr)	1 (sl.)	7	2†	-	†decr (?)
21	Fibr (a) decr (<200 mg)	1	4		-	†120 mg
	(b) incr (>540 mg)	5†			-	
22	AHF*: <60		10			
23	PTC*: <60		6		4	
25	Prothrombin Cons <90%	4	1	1†	4	†90%
26	Prothrombin Time*: <70	2	8			
27	Prothrombin*: <70	3	7			
28	Proconvertin*: <65	4	5	1†	-	†66
29	Proaccelerin*: <60	1	9	-		
	N P N (or B U N) mg/100 ml	10	0		-	†78 330

* percent of standard normal

TABLE LIX. Prothrombin Consumption Tests (2 stage) on a Case of Liver Carcinoma (Metastasis) Treated with Radio Gold (Au^{198})

Day of therapy	-1	4	8	13	19	22
Plasma (u/ml)	510	470	313	340	445	184
1 hr serum (Resid pro %)	23	1	2	2	20	45
Platelets x 10^3	243	280	235	420	158	48

TABLE LX Platelet Counts and Prothrombin Consumption in Three Dogs Injected with Radio Gold

DOG	I		II		III	
DOSE Au^{198} (mc/Kg)	20		10		5	
	Plat /mm^3	Pro Cons	Plat /mm^3	Pro Cons	Plat /mm^3	Pro Cons
Day: 0	354 500	96%	238 400		312 500	
2	217 500	93%	765 000			
4	242 500	95%			337 500	95%
6	45 000	0	339 600	80%	372 500	89%
8	22 500	14%	139 400	59%	135 000	97%
10	(died 9th day)		219 000	73%		40%
13			26 000	12%	77 500	33%
18			13 100	37%	145 000	66%

TAPLE LVIII G p VIII Plat l t P bl m i Ca with Pl m Cl ttl g Diffi lti s

1	79	80	81	82	( ) 83	84		85
2	B S	L F	R B	F M	E B	D H		C R
3	17	54	54	29	26	3		70
	M	M	M	M	F	F		M
	W	W	C	W	C	W		W
4	1954	1954	1954	1753	1953	1954	10 Jan 55	28 F b 55
5	?	1 wk	4 y	?	?	3 y	?	7
6	0	0	0	0	0	0 (7)	0	
7	+	±	+	±	+	+++	++	++
8	g	+	n g	eg	N T	+	N T	N T
9	2½	1	1½	5	N T	5	N T	N T
10	4½	4 3/4	5	10 ½	Ly i	39	Ly i	12
11	16	42	49	43	Lysl	4 h	Ly i	82
12	+++	Ly i	Lysl	++	Ly i	++++	Lysl	Ly i
13	0	+	+	0	+	0	+ (43)	+ (2 h)
14	31	89	90	9	128	240	36	152
15	0	15	43	50	N T	100	6	N T
16	1	1	20	29	N T	5	1	N T
17	150	100	100	100	N T	250	N T	N T
18	N T	N T	N T	N T	N T	N T	N T	N T
19	i	13	e 13	in	s 13	n m	ee 13	s 13
20	n m	N T	n m	o m	N T	m	m	m
21	320	250	560	340	200	410	20	40
22	*100*	*100*	*100*	*100*	*N T*	*100*	*100*	*N T*
23	20	60	20	50	20	90	100	N T
24	N T	N T	N T	N T	N T	0	200%	tilysl
25	90	95	95	93	N T	43	41	0
26	N T	34	53	20	23	1	7	24
27	41	25	30	44	43	113	78	91
28	43	2	25	57	59	108	79	100
29	45	40	40	40	80	0	10	25
30	LIVER DISORDERS					C g	C l m f P t t	
†	, un espo si e to it.K					1 k of	with circ lati g	
	Ja d, Ja d,					A G	Fib in lysin	

† I m mbe i g of t t am in T bl LII

TABLE LXIII E p lm t 59 P oth mbin (lu t) Activ tion by Stypv n and C ph lin in th Pres nc of V l u F ct r Defi len i Optim l C ln li te t nd ptim l additio of f t st t d Fib in g n cl tting time (s) with incubat t ½ l min and t ptim l period (min) 26 C

TEST NO	FACTOR DEFICIENCY	A G ADDED	STYPVEN ADDED	CEPHALIN ADDED	INCUBATION PERIOD (min): ½	1	optim l
1	AHF	+	+	+	5	24 2	23 2 (2)
2	PTC	+	+	+		34 5	22 6 (5)
3	Hag	+	+	+	29 7		17 2 (3)
4	H g	+	+	+	24 6		23 7 (3)
5	VII	+	+	+	26 4		23 1 (2)
6	AcG		+	+	94 9		32 8 (4)
7	AHF	+		+		167	23 4 (11)
8	AHF	+	+			350 7	248 4 (10)
9	PTC	+	+			720	501 (10)
10	H g	+	+			730	232 2 (20)
11	VII	+	+		203 3		76 5 (14)
12	H g		+	+	175 2		111 3 (19)
13	(H g +)		+	+	177 5		118 9 (19)
14	(A G+)	+	+	+	25 4		21 (2)

H g f e A G (e te t)

TABLE LXI Thrombin Formation 2 stage tests Various Thromboplastic Agents with or without AHF Prothrombin and other factors constant

EXPT NO.	AGENT +Ca, AcG	INITIAL RATE AHF	INITIAL RATE AHF+	OPTIMUM PERIOD AHF	OPTIMUM PERIOD AHF+	THROMBIN YIELD (%) AHF	THROMBIN YIELD (%) AHF+
1	Tpln	fast	fast	short	short	100	99
2	Ceph	very slow	slow	long	short	66	95
3	Sbp		"		short*	10	73*
4	Eap				(long) *	2	9*
5	Plat				short	75*	92
6	Rbc				short	40	100
7	0	negligibl activation in the control				0	0

Technical details given in text Experiment 56

TABLE LXII Bioassay of Prothromboplastin by Stypven 2 stage method Standard[1] = brain cephalin Unknown = Eap 26°C

TEST NO	AGENT TESTED	REL STR	FINAL CONC µg/ml	INCUBATION PERIOD: 1 min	INCUBATION PERIOD: optimal (min)	
1	Ceph	100%	30 0	17 8	16 8	(2')
2		10%	3 0	34 0	20 4	(4)
3		8"	2 4	36 1	20 5	(6')
4		6"	1 8	46 0	21 1	(13')
5		4"	1 2	52 0	22 5	(15)
6		2%	0 6	77 8	25 3	(17)
7		1%	0 3	127 1	54 3	(20')
8	0 (control)	0	0	351	284"	(10')
9	E p	1:1	400	69 8	23 4	(11)
10	Eap	1 2	200	82 5	27 9"	(15')

APPENDIX III

BIBLIOGRAPHY

TABLE LXIV Experiment 59 IV Prothrombin Activation in Stuart deficient Eluate Optimal Ca and AcG Effects of adding Stypven cephalin thromboplastin AHF and Stuart factor preparations (see text) Fibrinogen clotting times (sec) with incubate after 1 min and at optimum (min.) 26°C

TEST NO	AGENTS TESTED	FACTORS ADDED	INCUBATION PERIOD 1 min	opt (min)	THROMBIN YIELD (*)
1	*Stypven*		*1800*	*1800 (7)*	*trace*
2	*Stypven*	*Stuart (A)*	*258 2"*	*216 1 (5')*	*trace*
3	Stypven ceph.		59 3	27 4 (4)	52
4	Stypven ceph	Stuart (A)	22 4	16 8 (5')	100
5	Stypven ceph	Stuart (B)	24 7	19 9 (4)	89
6	Stypven tpln		64 3"	24 4 (7)	64
7	Stypven tpln	Stuart (A)	22 0	17 0 (5')	100
8	Tpln		184 8"	24 0 (11')	65
9	Tpln	Stuart (A)	30 8	17 4 (5)	98
10	Tpln	Stuart (B)	31 2	17 2 (4')	99
11	Ceph	AHF	315	26 3 (17)	55
12	Ceph	AHF Stuart (A)	73 6	16 0 (5')	104

TABLE LXV Various Prothromboplastins tested (2 stage) *in Stypven activation of prothrombin (eluate)*

TEST NO	AGENT TESTED	OPTIMAL TEST C T	INC PERIOD
1	Ceph	23 0	2 min
2	Sbp	22 2	3 min
3	Plat	20 6"	4 min
4	Rbc	20 9	3 min
5	Eap	23 4	11 min
6	0	>240	

BIBLIOGRAPHY

1 Ackroyd J F Brit. Med. Bull 11: 28 35 1955 Platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura with a note on platelet groups

2 Adamis D H S Size and D M Kimball J. Lab. Clin. Med 47: 320 325 1956 The proconvertin test a simplified method and its application to the study of anticoagulant processes

3 Addis T J. Pathol. Bacteriol 15 427 452 1910 11 The pathogenesis of hereditary hemophilia

4 Aggeler P M and S P Lucia Proc. Soc. Exptl. Biol. Med 38: 11 16 1938 Studies of some variables affecting prothrombin times

5 Aggeler P M S G White M B Glendening E W Page T B Leake and G Bates Proc. Soc. Exptl. Biol. Med 79 692 694 1952 Plasma thromboplastin component (PTC) deficiency; a new disease resembling hemophilia

6 Alexander B in Blood cells and plasma proteins, their state in nature J L Tullis ed Academic Press New York 1953 Chapter 2 pp 75 92 Some biochemical physiological and pathological aspects of the coagulation mechanism

7 New Engl. J. Med 252: 432 442; 484 494 526 535 1955 Coagulation hemorrhage and thrombosis

8 R L Goldstein and G Landwehr J. Clin. Invest 29 881-895 1950 The prothrombin conversion accelerator of serum (SPCA) its partial purification and its properties compared with serum Ac globulin

9 and C D Cook J. Clin. Invest 30: 596 608 1951 Congenital SPCA deficiency a hitherto unrecognized defect with hemorrhage rectified by serum and serum fractions

10 Alexander B R L Goldstein L Rich A G LeBolloch L K Diamond and W Borges Blood 9 843 865 1954 Congenital afibrinogenemia a study of some basic aspects of coagulation

11 Alexander B and G Landwehr Am. J. Physiol 159: 322 331 1949 Evolution of a prothrombin conversion accelerator in stored human plasma and prothrombin fractions

12 Alkjaersig N T Abe and W H Seegers Am. J. Physiol 181: 304 308 1955 Purification and quantitative determination of platelet factor 3

13 Alkjaersig N T Abe S A Johnson and W H Seegers Am. J Physiol 182: 443 446 1955 An accelerator of prothrombin activation derived from prothrombin

37 Bell W N Blood 6 1119 1203 1951 Coagulation defect due to anticoagulant possessing antithromboplastic and anti thrombic properties probably heparin
38 and H G Alton Nature 174 880 881 1954 A brain ex tract as a substitute for platelet suspensions in the throm boplastin generation test
39 Bendien W M and S van Creveld Acta Brevia Neerl, Physiol Pharmacol, Microbiol 5 135 138 1935 Investigations on hemophilia
40 Bergsagel D E and C Hougie Brit, J, Haematol 2 113 129 1956 Intermediate stages in the formation of blood thromboplastin
41 Berman H J G P Fulton and B R Lutz Federation Proc 15 17 1956 (Abstr) Platelets in peripheral circulation of the hamster
42 Bernfeld P and J S Nisselbaum Federation Proc, 15, 220 1956 (Abstr) Reaction of human β-lipoglobulin with macromolecular polysulfate esters
43 Bessis M Blood 5 1083 1098 1950 Studies in electron mi croscopy of blood cells
44 and H Burstein Rev, d hematol 3 48 68 69 91 1948 Études sur les thrombocytes au microscope electronique Etudes sur la physiologie des thrombocytes (revue gen erale et travaux personnels)
45 Best C H C Cowan and D L MacLean J, Physiol 92 20 31 1938 Heparin and the formation of white thrombi
46 Bidwell E Brit, J, Haematol 1 35 45 1955 The purification of bovine antihaemophilic globulin
47 Biggs R Nature 170 280 1952 Plasma thromboplastin
48 and A S Douglas J, Clin, Pathol 6 23 29 1953 The thromboplastin generation test
49 and R G MacFarlane J, Physiol 119 89 101 1953 The formation of thromboplastin in human blood
50 J, Physiol, 122 554 569 1953 The action of thrombo plastic substances
51 J V Dacie W R Pitney C Merskey and J R O Brien Brit, Med, J (2) 1378 1382 1952 Christmas disease a condition previously mistaken for hemophilia
52 Biggs R and R G MacFarlane Human blood coagulation and it disorders C C Thomas Springfield 1953 406 pp
53 Billing W M J, Pharmacol, Exptl, Therap 38 173 196 1930 The action of the toxin of crotalus adamanteus on blood clotting
54 Bizzozero G Virchow s Arch, pathol, Anat, u Physiol 90 267 332 1882 Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombo e und der Blutgerinnung
55 Bordet J Bull, Johns Hopkins Ho p 32 213 218 1921 The theories of blood coagulation
56 and L Delange Ann, inst, Paste r 26 657 674 1912 La coagulation du sang et la genese de la thrombine
57 Ann, inst, P steur 26 737 766 1912 I a réaction des plaquettes avec le serum

14 Allen J G and discussants in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 5: 213 246 1952 The pathogenesis of irradiation hem orrhage
15 Allen J G P V Moulder R M Elghammer B J Grossman C L McKeen M Sanderson W Egner and J M Cros bie J. Lab. Clin. Med 34: 473-476 1949 Protamine titration as an indication of a clotting defect in certain hemorrhagic states
16 Anderson A Proc. (Roy.) Soc. Glasgow 1: 200 206 1841-1844 Note on the state in which fibrin exists in the blood
17 Andral G Essai d'hématologie pathologique Fortin Masson Paris 1843 186 pp (p 32)
18 Apitz K Z. ges. exptl. Med 101: 552 584 1937 Uber Profibrin
19 Arthus M and C Pages Arch. physiol. norm. et pathol Ser 5 2: 739 746 1890 Nouvelle théorie chimique de la coag ulation du sang
20 Astrup T Acta Physiol. Scand 7: Suppl XXI 1-121 1944 Biochemistry of blood coagulation
21 - - - - and S Darling Acta Physiol. Scand 4: 293 308 1942 Measurement of properties of antithrombin
22 - Acta Physiol. Scand 5: 13-30 1943 Antithrombin and heparin
23 Auer J Am. J. Med. Sci 186: 776-794 1933 Structure and function of filaments produced by living red corpuscles
24 Aynaud M Le globulin des mammiferes These Paris 1909 243 pp
25 -- -- Ann. inst. Pasteur 25: 56 78 1911 Le globulin de l'homme
26 - in Traité du Sang A Gilbert and M Weinberg eds J B Balliere & fils Paris 1: 410 1913
27 - - - and P Jeantet in ibid p 442
28 Baer E Can. J. Biochem. Physiol 34: 288 304 1956 The syn thesis of glycerol phosphatides
29 - - J Maurukas and M Russell J. Am. Chem. Soc 74: 152 157 1952 Synthesis of enantiomeric α cephalins
30 Bailey K F R Betrelheim L Lorand and W R Middlebrook Nature 167 233-234 1951 Action of thrombin in clot ting of fibrinogen
31 Baserga A Le Sang 25: 742 751 1954 Les facteurs plaquet taires
32 Bayne-Jones S Am. J. Physiol 30 74 79 1912 13 The pres ence of prothrombin and thromboplastin in the blood plate lets
33 Bedson S P J. Pathol. Bacteriol 25: 94 104 1922 Blood platelet anti serum its specificity and role in the exper imental production of purpura
34 --- J. Pathol. Bacteriol 26: 145 155 1923 An enquiry into the genesis of the mammalian blood platelet
35 -- -Lancet (2): 1117-1119 1924 The effect of splenectomy on the production of experimental purpura
36 Bell H J XIX Internat. Physiol. Congr., Montreal (Abstr) pp 200 201 1953 The chemistry of heparin

76 Chargaff E J. Biol. Chem 155 387 399 1944 The thrombo plastic activity of tissue phosphatides
77 J. Biol. Chem 160: 351 359 1945 Cell structure and the problem of blood coagulation
78 F Bancroft and M Stanley-Brown J. Biol. Chem 116 237 251 1936 *Studies on the chemistry* of blood coagu lation III The chemical constituents of blood platelets and their role in blood clotting with remarks on the ac tivation of clotting by lipids
79 Chargaff E and S S Cohen J. Biol. Chem 129 619 628 1939 On lysophosphatides
80 Chargaff E and K B Olson J. Biol. Chem 122 153 167 1937 1938 Studies on the chemistry of blood coagulation VI Studies on the action of heparin and other *anticoagulants* The influence of protamine on the anticoagulant effect in vivo
81 Chargaff E and R West J. Biol. Chem 166 189 197 1946 The biological significance of the thromboplastic protein of blood
82 Chargaff E and M Ziff J. Biol. Chem 138 787 788 1941 Co agulation of fibrinogen by simple organic substances as a model of thrombin action
83 and S S Cohen J. Biol. Chem 136 257 264 1940 Studies on the chemistry of blood coagulation X The re action between heparin and the thromboplastic factor
84 Charles A F A M Fisher and D A Scott Trans. Roy. Soc. Can 28 49 54 1934 A blood coagulant from beef lung
85 Christie R V H W Davies and C P Stewart Quart. J. Med 20: 481 498 1927 Studies in blood coagulation and haemo philia II Observations on haemic functions in haemo philia
86 Clowes G H A Am. J. Physiol 42 610 611 1916 17 On the mechanism of blood coagulation
87 Cohen S S and E Chargaff J. Biol. Chem 136 243 256 1940 Studies on the chemistry of blood oagulation IX The thromboplastic protein from lungs
88 J. Biol. Chem 139 741 752 1941 Studies on the chem istry of blood coagulation XIII The phosphatide constitu ents of the thromboplastic protein from lungs
89 Cohn F J Physiol. Rev 5 349 437 1925 The physical chem istry of the proteins
90 Conley C L R C Hartmann and W I Morris (II) J. Clin. In vest 28 340 352 1949 Clotting behavior of human platelet free plasma II Evidence for existence of plasma thromboplastin
91 Conley C L and W I Morse Am. J. Med. Sci 215 158 169 1948 Thromboplastic factors in the estimation of pro thrombin concentration
92 Conley C L O D Ratnoff C E Ellicott and R C Hartmann J. Clin. Invest 29 1182 1188 1950 Studies on the ini tiation of blood coagulation II An anticoagulant inhibiting the activation of a plasma thromboplastic factor
93 Copley A L and T P Robb Am. J. Clin. Pathol 12 36_ 371 416 423 563 570 1942 *Studies on platelets I The*

58 Bordet J and O Gengou Ann. Inst. Pasteur 18: 26 40 1904 Recherches sur la coagulation du sang: contribution a l'etude du plasma fluoré
59 Bowman D E Arch. Biochem 16 109 113 1948 Further differentiation of bean trypsin inhibitory factor
60 Boyles P W J H Ferguson and P H Muehlke J. Gen. Physiol 34: 493 513 1951 Mechanisms involved in fibrin formation
61 Brambel C E A H Corwin and V A Capone Am. J. Med. Sci 230: 276 292 1955 Mechanism of action of heparin as an etiological basis for homeostasis (hemorrhage and thrombosis)
62 Brecher G and E P Cronkite J. Appl. Physiol 3 365 377 1950 Morphology and enumeration of human blood platelets
63 Brinkhous K M Am. J. Med. Sci 198 509 516 1939 A study of the clotting defect in hemophilia the delayed formation of thrombin
64 - - Proc. Soc. Exptl. Biol. Med 66: 117 120 1947 Clotting defect in hemophilia: deficiency in a plasma factor required for platelet utilization
65 - Le Sang 25 738 741 1954 Plasma antihemophilic factor Biological and clinical aspects
66 - R D Langdell G D Penick and J B Graham J. Am Med. Assoc 154 481 486 1954 Newer approaches to the study of hemophilia and hemophilioid states
67 Brinkhous K M H P Smith E D Warner and W H Seegers Am. J. Physiol 125: 683 687 1939 The inhibition of blood clotting: an unidentified substance which acts in conjunction with heparin to prevent the conversion of prothrombin to thrombin
68 Buchanan A Proc. (Roy.) Soc. Glasgow 1: 131-145 1841 44 On the fibrin contained in the animal fluids the mode in which it coagulates and the transformations which it undergoes
69 Proc. (Roy.) Soc. Glasgow 2: 16 22 1845 On the coagulation of the blood and other fibriniferous liquids (Repr J. Physiol 2: 158 163 1879)
70 Budtz Olsen O E Clot retraction C C Thomas Springfield 1951 149 pp
71 Cekada E B Am. J. Physiol 78: 512 532 1926 The preparation and properties of prothrombin
72 Chaptal J A Elemens de chymie Deterville Paris 3 vols 1790 361 448; 495 pp (4th Amer ed trans J Woodhouse B and J Kite Philadelphia 2 vols 1807 339; 454 pp)
73 Chargaff E J. Biol. Chem 121 175 186 1937 Studies on the chemistry of blood coagulation IV Lipid inhibitors of blood clotting occurring in mammalian tissue
74 - J. Biol. Chem 125 661-670 1938 The protamine salts of phosphatides with remarks on the problem of lipoproteins
75 - - - J. Biol. Chem 125 671-676 1938 Studies on the chemistry of blood coagulation VII Protamines and blood clotting

76 Chargaff E J. Biol. Chem 155 387 399 1944 The thrombo plastic activity of tissue phosphatides
77 J. Biol. Chem 160: 351 359 1945 Cell structure and the problem of blood coagulation
78 F Bancroft and M Stanley Brown J. Biol. Chem 116 237 251 1936 Studies on the chemistry of blood coagu lation III The chemical constituents of blood platelets and their role in blood clotting with remarks on the ac tivation of clotting by lipids
79 Chargaff E and S S Cohen J. Biol. Chem 129 619 628 1939 On lysophosphatides
80 Chargaff E and K B Olson J. Biol. Chem 122 153 167 1937 1938 Studies on the chemistry of blood coagulation VI Studies on the action of heparin and other anticoagulants The influence of protamine on the anticoagulant effect in vivo
81 Chargaff E and R West J. Biol. Ch m 166 189 197 1946 The biological significance of the thromboplastic protein of blood
82 Chargaff E and M Ziff J. Biol. Chem 138 787 788 1941 Co agulation of fibrinogen by simple organic substances as a model of thrombin action
83 and S S Cohen J. Biol. Chem 136 257 264 1940 Studies on the chemistry of blood coagulation X The re action between heparin and the thromboplastic factor
84 Charles A F A M Fisher and D A Scott Trans. Roy. Soc. Can 28 49 54 1934 A blood coagulant from beef lung
85 Christie R V H W Davies and C P Stewart Quart. J. Med 20 481 498 1927 Studies in blood coagulation and haemo philia II Observations on haemic functions in haemo philia
86 Clowes G H A Am. J. Physiol 42 610 611 1916 17 On the mechanism of blood coagulation
87 Cohen S S and E Chargaff J. Biol. Chem 136 243 256 1940 Studies on the chemistry of blood coagulation IX The thromboplastic protein from lungs
88 J. Biol. Chem 139 741 752 1941 Studies on the chem istry of blood coagulation XIII The phosphatide constitu ents of the thromboplastic protein from lungs
89 Cohn E J Physiol. Rev 5 349 437 1925 The physical chem istry of the proteins
90 Conley C L R C Hartmann and W I Morris (II) J. Clin In vest 28 340 352 1949 Clotting behavior of human platelet free plasma II Evidence for existence of plasma thrombopla tin
91 Conley C L and W I Morse Am. J. Med. Sci 215 158 169 1948 Thromboplastic factors in the estimation of pro thrombin concentration
92 Conley C L O D Ratnoff C E Ellicott and R C Hartmann J. Clin. Invest 29 1182 1188 1950 Studies on the ini tiation of blood coagulation II An anticoagulant inhibiting the activation of a plasma thromboplastic factor
93 Copley A L and T P Robb Am. J Clin. Pathol 12 362 371 416 423 563 570 1942 Studies on platelets I The

58 Bordet J and O Gengou Ann. Inst. Pasteur 18: 26 40 1904 Recherches sur la coagulation du sang contribution a l'etude du plasma fluore
59 Bowman D E Arch. Biochem 16 109 113 1948 Further differentiation of bean trypsin inhibitory factor
60 Boyles P W J H Ferguson and P H Muehlke J. Gen. Physiol 34 493 513 1951 Mechanisms involved in fibrin formation
61 Brambel C E A H Corwin and V A Capone Am. J. Med. Sci 230: 276 292 1955 Mechanism of action of heparin as an etiological basis for homeostasis (hemorrhage and thrombosis)
62 Brecher G and E P Cronkite J. Appl. Physiol 3: 365 377 1950 Morphology and enumeration of human bloodplatelets
63 Brinkhous K M Am. J. Med. Sci 198: 509-516 1939 A study of the clotting defect in hemophilia the delayed formation of thrombin
64 - - Proc. Soc. Exptl. Biol. Med 66: 117-120 1947 Clotting defect in hemophilia: deficiency in a plasma factor required for platelet utilization
65 - Le Sang 25 738 741 1954 Plasma antihemophilic factor Biological and clinical aspects
66 R D Langdell G D Penick and J B Graham J. Am Med. Assoc 154 481 486 1954 Newer approaches to the study of hemophilia and hemophilioid states
67 Brinkhous K M H P Smith E D Warner and W H Seegers Am. J. Physiol 125: 683 687 1939 The inhibition of blood clotting: an unidentified substance which acts in conjunction with heparin to prevent the conversion of prothrombin to thrombin
68 Buchanan A Proc. (Roy.) Soc. Glasgow 1: 131 145 1841 44 On the fibrin contained in the animal fluids the mode in which it coagulates and the transformations which it undergoes
69 - Proc. (Roy.) Soc. Glasgow 2: 16 22 1845 On the coagulation of the blood and other fibriniferous liquids (Repr J. Physiol 2: 158 163 1879)
70 Budtz Olsen O E Clot retraction C C Thomas Springfield 1951 149 pp
71 Cekada E B Am. J. Physiol 78: 512-532 1926 The preparation and properties of prothrombin
72 Chaptal J A *Elemens de chymie* *Deterville Paris* 3 vols 1790 361 448; 495 pp (4th Amer ed trans J Woodhouse B and J Kite Philadelphia 2 vols 1807 339; 454 pp)
73 Chargaff F J. Biol. Chem 121: 175 186 1937 Studies on the chemistry of blood coagulation IV Lipid inhibitors of blood clotting occurring in mammalian tissue
74 -- J. Biol. Chem 125: 661-670 1938 The protamine salts of phosphatides with remarks on the problem of lipoproteins
75 - - J. Biol. Chem 125 671 676 1938 Studies on the chemistry of blood coagulation VII Protamines and blood clotting

109 Duke W W J. Am. Med. Assoc 55: 1185 1192 1910 The relation of blood platelets to hemorrhagic disease
110 Eagle H J. Gen Physiol 18: 531 545 1935 Studies in blood coagulation 1 The role of prothrombin and of platelets in the formation of thrombin
111 - J. Exptl. Med 65: 613 639 1937 The coagulation of blood by snake venoms and its physiologic significance
112 and T N Harris J. Gen. Physiol 20: 543 560 1937 Studies in blood coagulation V The coagulation of blood by proteolytic enzymes (trypsin papain)
113 Eberth J C and C Schimmelbusch Virchow s Arch. Pathol Anat. u. Physiol 103: 39 87 1886 Experimentelle Untersuchungen über Thrombose
114 Edsall J T Advances in Protein Chem 3 383 479 1947 The plasma proteins and their fractionation
115 and W F Lever J. Biol. Chem 191: 735 756 1951 Effects of ions and neutral molecules on fibrin clotting
116 Emmel V E Am. J. Anat 16: 127 205 1914 Concerning certain cytological characteristics of the erythrocytes in the pig embryo and the origin of non nucleated erythrocytes by a process of cytoplasmic constriction
117 Erickson B N H H Williams I Avrin and P Lee J. Clin Invest, 18: 81 85 1939 The lipid distribution of human platelets in health and disease
118 Erkelens A D De plaats der thrombocyten in het stollings proces Thesis Rotterdam Boerman & Zoon 1956 174 pp
119 Fantl P and M Nance Nature 158: 708 709 1946 Acceleration of thrombin formation by plasma component
120 - Med. J. Australia 2: 125 128 1946 An acquired haemorrhage disease in a female due to an inhibitor of blood coagulation
121 Fenichel R L and W H Seegers Am. J. Physiol 181 19 20 1955 Bovine platelets serotonin and the retraction of bovine plasma clots
122 Ferguson J H Trans. Roy. Soc. S. Africa 18: 317 323 1930 Living human blood cells under the dark ground microscope
123 Proc. Soc. Exptl. Biol. Med 31: 929 930 1934 Cephalin content of prepared fibrinogen and prothrombin solutions
124 Am. J. Physiol, 108 670 682 1934 Observations on the alterations of blood platelets as a factor in coagulation of the blood
125 Physiol. Rev 16: 640 670 1936 The blood calcium and the calcium factor in blood coagulation
126 Am. J. Physiol. 117: 587 595 1936 An experimental analysis of coagulant activation
127 J. Lab. Clin. Med 22: 462 466 1937 Observations on living *mesenteric capillaries*
128 Am. J. Physiol. 119: 755 762 1937 An intermediary complex in blood coagulation
129 Am. J. Physiol 123: 341 348 1938 Quantitative relationships of calcium and cephalin in experimental thrombin formation

method of Vilarino and Pimentel and a new direct method of counting the blood platelets II The effect of heparin on the platelet count in vitro III The effect of heparin in vivo on the platelet count in mice and dogs
94 Correll J L Lyth S Long and J Vanderpoel Am J. Physiol 163 537 544 1952 Some physiologic responses to 5 hydroxytryptamine creatinine sulfate
95 Cramer W and H Pringle Quart. J. Exptl. Physiol 6 1 11 1913 On the coagulation of blood
96 Cronkite E P Radiology 56: 661 669 1951 The diagnosis prognosis and treatment of radiation injuries produced by atomic bombs
97 G J Jacobs G Brecher and G Dillard Am. J. Roentgenol. Radium Therapy 67 796 804 1952 The hemorrhagic phase of the acute radiation syndrome due to exposure of the whole body to penetrating ionizing radiation
98 Dale H H and G S Walpole Biochem. J 10 331 362 1916 Some experiments on factors concerned in the formation of thrombin
99 (De) Fourcroy A F (a) System de connaissances chimiques, et de leurs applications aux phenomènes de la nature et de l art Paris 10 vols 1801 (b) Trans W Nicholson Cadell & Davies London 1804
100 Dekhuyzen M C Anat. Anzeig 19 529 540 1901 Über die Thrombocyten (Blutplättchen)
101 De Nicola P Texas Repts. Biol. Med 11 3 16 1953 Research on the new clotting factors
102 Denis P -S Memoire sur le sang considere quand il est fluide pendant qu'il se coagule et lorsqu'il est coagule; suive d'une notice sur l'application de la methode d'experimentation par les sels a l'étude des substances albumoides (Acad d Sci 20 dec 1858) J B Bailliere et Fils Paris 1859 208 pp
103 Deuel H J Jr The lipids, their chemistry and biochemistry Vol I Chemistry Chap V Chemistry of the phosphatides and cerebrosides (2) Historical development pp 407 408 Interscience Publ New York 1951 982 pp
104 Deutsch E S A Johnson and W H Seegers Circulation Res 3 110 115 Differentiation of certain platelet factors related to blood coagulation
105 De Vries S I H K Kettenborg and E T van der Pol Ned Tijdschr. Geneesk. 99 2967 2974 1955 Verband tussen een factor uit rode bloedcellen en de vorming van thromboplastine
106 Donne A Compt. rend. 14: 366 368 1842 De l'origine des globules du sang de leur mode de formation et de leur fin
107 Douglas S R and L Colebrook Lancet (2): 180 183 1916 On the advantage of using a broth containing trypsin in making blood cultures
108 Duckert F P Fluckiger M Matter and F Koller Proc. Soc Exptl. Biol. Med 90 17 22 1955 Clotting factor X Physiologic and physico chemical properties

109 Duke W W J. Am. Med. Assoc 55: 1185 1192 1910 The relation of blood platelets to hemorrhagic disease
110 Eagle H J. Gen Physiol 18: 531 545 1935 Studies in blood coagulation I The role of prothrombin and of platelets in the formation of thrombin
111 J. Exptl. Med 65: 613 639 1937 The coagulation of blood by snake venoms and its physiologic significance
112 and T N Harris J. Gen. Physiol 20 543 560 1937 Studies in blood coagulation V The coagulation of blood by proteolytic enzymes (trypsin papain)
113 Eberth J C and C Schimmelbusch Virchow's Arch. Pathol Anat. u. Physiol 103 39 87 1886 Experimentelle Untersuchungen uber Thrombose
114 Edsall J T Advances in Protein Chem 3 383 479 1947 The plasma proteins and their fractionation
115 and W F Lever J. Biol. Chem 191 735 756 1951 Effects of ions and neutral molecules on fibrin clotting
116 Emmel V E Am. J. Anat 16: 127 205 1914 Concerning certain cytological characteristics of the erythrocytes in the pig embryo and the origin of non nucleated erythrocytes by a process of cytoplasmic constriction
117 Erickson B N H H Williams I Avrin and P Lee J. Clin Invest. 18 81 85 1939 The lipid distribution of human platelets in health and disease
118 Erkelens A D De plaats der thrombocyten in het stollings proces Thesis Rotterdam Boerman & Zoon 1956 174 pp
119 Fantl P and M Nance Nature 158: 708 709 1946 Acceleration of thrombin formation by plasma component
120 Med. J. Australia 2: 125 128 1946 An acquired haemorrhage disease in a female due to an inhibitor of blood coagulation
121 Fenichel R L and W H Seegers Am. J. Physiol 181 19 20 1955 Bovine platelets serotonin and the retraction of bovine plasma clots
122 Ferguson J H Trans. Roy. Soc. S. Africa 18: 317 323 1930 Living human blood cells under the dark ground microscope
123 Proc. Soc. Exptl. Biol. Med 31 929 930 1934 Cephalin content of prepared fibrinogen and prothrombin solutions
124 Am. J. Physiol. 108 670 682 1934 Observations on the alterations of blood platelets as a factor in coagulation of the blood
125 Physiol. Rev 16: 640 670 1936 The blood calcium and the calcium factor in blood coagulation
126 Am. J. Physiol. 117: 587 595 1936 An experimental analysis of coagulant activation
127 J. Lab. Clin. Med 22: 462 466 1937 Observations on living mesenteric capillaries
128 Am. J. Physiol. 119 755 762 1937 An intermediary complex in blood coagulation
129 Am. J. Physiol 123 341 348 1938 Quantitative relationships of calcium and cephalin in experimental thrombin formation

130 Ferguson J H J. Lab. Clin. Med 24 273-282 1938 A standardized procedure for the study of coagulation reactions (in vitro)

131 - - -Proc. Soc. Exptl. Biol. Med 42: 33 37 1939 Heparin and plasma albumin in relation to thromboplastic action of trypsin cephalin and brain extracts

132 Am. J. Physiol 126: 669-672 1939 The clotting of hemophilic plasma by thromboplastic enzyme

133 - - Ann. Rev. Physiol 2 71 108 1940 Blood: coagulation biophysical characters and formed elements

134 Am. J. Physiol. 130: 759 770 1940 The action of heparin serum albumin (crystalline) and salmine on blood clotting mechanisms (in vitro)

135 -- J. Gen. Physiol 25 607-616 1942 The flocculation maximum (pH) of fibrinogen and some other blood clotting reagents (Relative turbidimetry with the Evelyn photo electric colorimeter)

136 - Science 97: 319 322 1943 A new blood clotting theory

137 - Proc. Soc. Exptl. Biol. Med 52: 243 245 1943 Assay of tryptases by lysis of fibrinogen

138 - in Colloid Chemistry: theoretical and applied J Alexander ed Reinhold New York 5 951 957 1944 The modern outlook on coagulation

139 -- J. Elisha Mitchell Sci. Soc 61: 148 156 1945 Dark field microscopy of surface phenomena in phospholipid films and in formed elements of blood and bone marrow

140 - Ann. Rev. Physiol 8: 231 262 1946 Blood coagulation thrombosis and hemorrhagic disorders

141 Am. J. Med 3: 67 77 1947 Mechanisms of blood coagulation

142 - Ann. N. Y. Acad. Sci 49: 486 500 1948 A review of some basic facts of blood coagulation

143 -- - Quart. of Phi Beta Pi 44 279 287 1948 *Nomenclature of parenteral proteases*

144 -- in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2: 53 58 1949 Thromboplastic mechanism of blood clotting

145 - in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2: 92 94 1949 Profibrin

146 -- in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2: 171 174 1949 Thrombin yields and thrombin stability

147 in Blood cells and plasma proteins J L Tullis ed Academic Press New York 1953 Chap 3 pp 93 120 Some experiences with blood clotting mechanisms

148 - G A Andrews and M Brucer Proc. Soc. Exptl. Biol Med 80 541 545 1952 Blood clotting studies on dogs internally irradiated with radio gold

149 Ferguson J H and B N Erickson Am. J. Physiol 126: 661 668 1939 The coagulant action of crystalline trypsin cephalin and lung extracts

150 Ferguson J H and B N Erickson Proc. Soc. Exptl. Biol. Med 40: 625 626 1939 Calcium and cephalin in relation to the clotting power of crystalline trypsin
151 Ferguson J H and A J Glazko J. Lab. Clin. Med 26 1559 1564 1941 Heparin
151a Am. J. Physiol. 134: 47 53 1941 Heparin and natural antithrombin in relation to activation and assay of prothrombin
152 Ferguson J H J H Lewis and T Arends Blood 11 846 855 1956 Hemorrhagic disease with circulating inhibitors of blood coagulation: Anti AHF and anti PTC in eight cases
153 Ferguson J H J H Lewis and J W Fresh Yale J. Biol. Med 28: 253 264 1955 56 The two stage 'prothrombin assay in study of bleeding and clotting disorders
154 Ferguson J H and M J Patch Proc. Soc. Exptl. Biol. Med 93: 193 196 1956 Determinants of the so called prothrombin time
155 Ferguson J H and P H Ralph Am. J. Physiol 138: 648 651 1943 Ninhydrin crystalline papain and fibrin clot
156 Ferguson J H B L Travis and E B Gerheim Blood, 3, 1130 1160 1948 The activation of prothrombin with special reference to thromboplastic enzyme (tryptase)
157 Ferry J D in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2: 84 105 1949 Consideration of the structure and mechanism of forma tion of the fibrin clot
158 Physiol. Rev 34: 753 760 1954 Polymerization of fibrinogen
159 and P R Morrison J. Am. Chem. Soc. 69: 388 400 1947 Preparation and properties of serum and plasma proteins VIII The conversion of human fibrinogen under various conditions
160 Ferry J D and S Shulman J. Am. Chem. Soc 71 3198 3204 1949 The conversion of fibrinogen to fibrin I Influence of hydroxyl compounds on clotting time and clot opacity
161 Fidlar E and L B Jaques J. Lab. Clin. Med 33 1410 1423 1948 The effect of commercial heparin on the platelet count
162 Fischer A. Japan J. Exptl. Med 13: 223 242 1935 Studies on coagulation of the blood
163 Biochem. Z 278 320 325 1935 Gerinnungszeit und Konzentration des Gerinnungsstoffes
164 Fitzgerald M A and D F Waugh Arch. Biochem. and Biophys 58 431 452 1955 Characterization of a heparin cofactor obtained from bovine plasma Fraction I
165 Flynn J E and R W Coon Ann. Rev. Physiol 14 205 234 1952 Fundamentals of blood clotting
166 Am. J. Physiol 175 289 295 1953 Purification and isolation of certain intermediates formed prior to the activation of prothrombin
167 Flynn J E and E T Standley in Josiah Macy Jr Found Confs on Blood clotting and allied probl ms J E Flynn ed 2 137 140 1949 Chargaff s circulating thromboplastin

168 Folch J J. Biol. Chem 146: 35 44 1942 Brain cephalin a mixture of phosphatides Separation from it of phospha tidyl serine phosphatidyl ethanolamine and a fraction containing an inositol phosphatide

169 Fon o A Ztschr. klin. Med 119: 687 700 1932 Die Unter kuhlungs Zentrifugiermethode Ein neues Verfahren zur Gewinnung von plattchenfreiem Plasma ohne gerinnungs hemmende Zusatze als Beitrag zur Untersuchungs methodik der Blutgerinnung

170 --- Acta Haematol 8: 363 367 1952 Ueber die Wirkung des Hyalomers der Thrombocyten auf den Retraktionsvor gang

171 -and J Schwendener Die Thrombozyten des menschlichen Blutes und ihre Beziehung zum Gerinnungs und Throm bosevorgang H Huber Bern 1942 130 pp

172 Fresh J W J H Ferguson and J H Lewis Obstet. & Gynecol 7 117 127 1956 Blood clotting studies in parturient women and the newborn

173 Fresh J W J H Ferguson C Stamey F M Morgan and J H Lewis Pediat. 19 241 251 1957 Blood prothrombin proconvertin and proaccelerin in normal infancy: ques tionable relationships to vitamin K

174 Freund E Wien. med Jahrb 1886 pp 46-48 Ein Beitrag zur Kenntniss der Blutgerinnung

175 Frick P G Blood 10 691 706 1955 Acquired circulating anticoagulants in systemic collagen disease

176 Fuchs H J Biochem. Z 222 470 481 1930 Wichtige methodische Einzelheiten die Blutgerinnungsuntersuchungen sowei eine Isolierungsmethodik des physiologischen gerinnungshemmenden Faktors (Antithrombin) aus Blut und Gewebe

177 Klin Wochschr 9 243 245 1930 Eine neue Theorie über die Blutgerinnung

178 Z. Immunitatsforsch 69 305-312 1930 Herstellung eines reinen und stabilen Plasmas mittels einfachen Zentrifugierens aus Saugetierblut

179 Z. ges. exptl. Med 79 76 86 1931 Uber die Ursache der Zusammenzeihung des Blutkuchens

180 Z Immunitatsforsch 58 14 22 1928 Ueber proteo lytische Fermente im Serum XII Die Bedeutung des Komplementes bei der Blutgerinnung

181 Fuld E and K Spiro Hofmeister s Beitr. z chem. Physiol. u Pathol 5 171-190 1904 Der Einflusz einiger gerinnungshemmender Agentien auf das Vogel plasma

182 Furchgott R F Cold Spring Harbor Symposia Quant. Biol. 8 224 232 1940 Observations on the structure of red cell ghosts

183 Gamgee A J. Physiol 2 145 157 1879 Some old and new ex periments on the fibrin ferment

184 Gasser H S Am. J. Physiol 42 378 394 1917 The significance of prothrombin and of free and combined thrombin in blood serum

185 George W C and J H Ferguson J. Morphol 86 315 324 1950 The blood of gastropod molluscs
186 Gerheim E B and J H Ferguson Proc. Soc. Fxptl. Biol. Med 71 258 261 1949 Staphylocoagulation in plasmas of various animal species
187 and B L Travis Proc. Soc. Exptl. Biol. Med 66 525 527 1947 Activation of staphylocoagulase
188 Glanzmann E Jahrb. Kinderheilk 88 113 141 1918 Hereditare hamorrhagischer Thromboasthenie Ein Beitrag zur Pathologie der Blutplattchen
189 Glavind J Studies on the coagulation of crustacean blood Thesis Copenhagen Nyt Nordisk Forlag A Busck 1948 137 pp
190 Glazko A J and J H Ferguson J. Gen. Physiol 24 169 188 1940 Kinetics of thrombin inactivation as influenced by physical conditions trypsin and serum
191 Glazko A J and D M Greenberg Am. J. Physiol 128 399 407 1940 Mechanism of inhibiting effect of electrolytes and heparin on blood coagulation
192 Goddard C H Am. J. Physiol 35 333 339 1914 The effect of filtration through a Berkefeld filter upon the coagulability of oxalated plasma
193 Goldsmith D P and C W Mushett J. Biol Chem 211 169 181 1954 Studies of lipide anticoagulants II Isolation pro cedures
194 Gollub S F E Kaplan D R Meranze and H (S) Tuft Am. J Clin. Pathol 19: 1071 1075 1949 Critical adsorption levels and electrophoretic analyses of prothrombin free plasmas
195 Graham J B O H Lowry N Wahl and M K Priebat J. Exptl Med 102 307 318 1955 Mast cells as sources of tissue histaminase
196 Graham J B and E M Barrow Federation Proc 14 404 1955 (Abstr) Analysis of biphasic clotting curve on dilution of plasma
197 Graham J B J A Buckwalter L J Hartley and K M Brinkhous J. Exptl Med 90 97 111 1949 Canine hemophilia Observations on the course the clotting anomaly and the effect of blood transfusions
198 Hougie C E M Barrow and J B Graham J. Clin. Invest 36 485 496 1957 Stuart clotting defect I Segregation of an hereditary hemorrhagic state from the heterogeneous group heretofore called stable factor (SPCA procon vertin factor VII) deficiency
199 Graszberger A Folia Haematol 36 17 24 1928 Beitrag zur Kenntniss des Vorkommens von Pseudospirochaeten im menschlichen Blut
200 Gratia A and P A Levene J Biol. Chem 50 455 461 1922 The role of cephalin in blood coagulation
201 Green J R J. Physiol 8 354 377 1887 On certain points connected with the coagulation of the blood
202 Grun A and R Limpacher Ch m. Ber 60 151 156 1927 Kephalin

203 Guest M M and A G Ware Science 112 21 22 1950 Fibrinolytic activity of purified prothrombin

204 Hall C E J. Biol. Chem 179: 857-864 1949 Electron microscopy of fibrinogen and fibrin

205 Hammarsten O Pflügers Arch. ges. Physiol 30: 437 484 1882 83 Ueber den Faserstoff und seine Enstehung aus dem Fibrinogen

206 Hoppe Seyler's Z. Physiol. Chem 22 333 395 1896 Ueber die Bedeutung der loslichen Kalksalze fur die Faserstoffgerinnung

207 Harrington W J V Minnich J W Hollingsworth and C V Moore J. Lab. Clin. Med 38 1-10 1951 Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura

208 Harrington W J C C Sprague V Minnich C V Moore R C Ahlvin and R Dubach Ann. Internal Med 38: 433 469 1953 Immunological mechanisms in idiopathic and neonatal thrombocytopenic purpura

209 Hartmann R C and C L Conley J. Clin. Invest 31 685 691 1952 Studies on the initiation of blood coagulation III The clotting properties of canine platelet free plasma

210 and J S Lalley Bull. JohnsHopkins Hosp 85 231 244 1949 Studies on the initiation of blood coagulation I Relationship of platelets to clot promoting effect of glass surfaces

211 Haurowitz F and J Sladěk Hoppe Seyler's Z. physiol. Chem 173 233 234 1928 Über die chemische Zusammensetzung der Blutplattchen

212 Hawn C van Z and K R Porter J. Exptl. Med 86 285-292 1947 The fine structure of clots formed from purified bovine fibrinogen and thrombin a study with the electron microscope

213 Hayem G Compt. rend. soc. biol (1877) 6 s 4 97 110 1879 Note sur les caracteres et l evolution des hematoblastes chez les ovipares

214 Arch. physiol. norm. et pathol 2 s 5 692 734 1878; 6: 201-261 1879 *Recherches sur l'évolution des hematies dans le sang de l homme et des vertebres*

215 Compt. rend 123 894 896 1896 Du caillot non retractile: suppression de la formation du sérum sanguin quelques états pathologique

216 - L'hematoblaste, troisième element du sang (avant propose et annotations de L Rivet) Presses Univ de France Paris 1923 296 pp (*cf Hayem's original ed 1877*)

217 Haykraft J B Naunyn Schmiedeberg's Arch. exptl Pathol. Pharmakol 18 209 217 1884 Ueber die Einwirkung eines Secretes des officinellen Blutegels auf die Gerinnbarkeit des Blutes

218 Hewson W Experimental Inquiries (a) 2nd ed T Cadell London 1771 223 pp (1st ed 1771 different title) (b) 3rd ed *I II J Johnson III T Longman London 3 vols* 1772 1773 1777 (ed M Falconar) pp 223 239 144 (c) Sydenham Soc ed G Gulliver ed London 1846 360 pp

219 Hirose R S Am. J. Physiol 107 693 697 1934 The second phase of thrombin action: fibrin resolution
220 Hirshboeck J S J. Lab. Clin. Med 33: 347 355 1948 The effect of operation and illness on clot retraction; description of a new method
221 Hirschfeld L and R Klinger Biochem. Z 70 398 415 1915 Zur Frage der Cobragiftinaktivierung des Serums
222 Hjort P S I Rapaport and P A Owren Blood 10 1139 1150 1955 Evidence that platelet accelerator (platelet factor 1) is adsorbed plasma proaccelerin
223 Hörder M H Acta Haematol 13 235 241 1955 Isolierter Factor V Mangel bedingt durch einen spezifischen Hemmkorper
224 and G Sokal Acta Haematol 14 294 302 1955 Der Einfluss von Factor V auf die Plasma Thromboplastin bildung
225 Hougie C Brit. J. Haematol 1 213 222 1955 The activation of platelets by plasma
226 Brit. Med. Bull 11 16 20 1955 Circulating anticoagulants
227 - and M E Fearnley Acta Haematol 12: 1 10 1954 The nature and action of circulating anticoagulants
228 Howe P E Physiol. Rev 5: 439 476 1925 The function of the plasma proteins
229 Howell W H Am. J. Physiol 26: 453 473 1910 The preparation and properties of thrombin together with observations on antithrombin and prothrombin
230 Am. J. Physiol 29 187 209 1911 12 The role of antithrombin and thromboplastin (thromboplastic substance) in the coagulation of the blood
231 Am. J. Physiol 31: 1 21 1912 13 The nature and action of the thromboplastic (zymoplastic) substance of the tissues
232 Am. J. Physiol 32 264 265 1913 Rapid method of preparing thrombin
233 Am. J. Physiol 35: 474 482 1914 Prothrombin
234 Am. J. Physiol 40: 526 546 1916 Structure of the fibrin gel and theories of gel formation
235 Harvey Lectures 12 273 324 1916 17 The coagulation of blood
236 Proc. Inst. Med., Chicago 5: 139 163 1925 The problem of coagulation (Pasteur Lect)
237 Bull. Johns Hopkins Hosp 42 199 206 1928 The purification of heparin and its chemical and physiological reactions
238 Physiol. Rev 15 435 470 1935 Theories of blood coagulation
239 Bull. Johns Hopkins Hosp 76 295 301 1945 The isolation of thromboplastin from lung tissue
240 and E B Cekada Am. J. Physiol 78: 500 511 1926 The cause of the delayed clotting of hemophilic blood
241 Howell W H and D D Donahue J. Exptl. Med 65 177 203 1937 The production of blood platelets in the lungs
242 Howell W H and E Holt Am. J. Phy.iol 48 328 341 1918 Two new factors in blood coagulation heparin and pro antithrombin

243 Humphrey J H and R Jaques J. Physiol 124 305 310 1954 The histamine and serotonin content of the platelets and polymorphonuclear leucocytes of various species
244 Ivy A C P F Shapiro and P Melnick Surg, Gynecol Obstet 60 781 784 1935 The bleeding tendency in jaundice
245 Jackson D P E P Cronkite G J Jacobs and C F Behrens Am. J. Physiol 169: 208 217 1952 Prothrombin utilization in radiation injury
246 Jackson D P E P Cronkite G V Le Roy and B Halpern J Lab. Clin. Med 39 449 461 1952 Further studies on the nature of the hemorrhagic state in radiation injury
247 Jaques L B Biochem. J 32 1181 1184 1938 The nitrogen partition in blood clotting
248 J. Biol. Chem 133 445 451 1940 Heparinase
249 Acta Haematol 2 188 199 1949 Determination of heparin in blood
250 A F Charles and C H Best Acta Med. Scand Suppl 90 190 207 1938 The administration of heparin
251 Jaques L B E Fidlar E T Feldsted and A G MacDonald Can. Med Assoc. J 55 26 31 1946 Silicones and blood coagulation
252 Jaques L B and E Keeri Szanto Can. J. Med. Sci 30 353 359 1952 Heparinase II Distribution of enzyme in various tissues and its action on natural heparins and certain synthetic anticoagulants
253 Jaques L B and R A Mustard Biochem. J 34: 153 158 1940 Some factors influencing the anticoagulant action of heparin
254 Jaques L B E Napke and S W Levy Circulation Research 1 321 330 1953 The metachromatic activity of urine following the injection of heparin
255 Jaques L B and E T Waters J. Physiol 99 454 466 1941 The identity and origin of the anticoagulant of anaphylactic shock in the dog
256 Johnson J F and W H Seegers Techniques in blood coagulation used at Wayne University (Mimeo) 1953 76 pp
257 Johnson S A and C L Schneider Science 117 229 230 1953 The existence of antifibrinolysin activity in platelets
258 Jorpes E Heparin its chemistry, physiology and application in medicine Oxford Univ Press 1939 87 pp
259 Jurgens J Acta Haematol 14 57 62 1955 Factor VII–inhibitor a new physiological serum accelerator inactivation principle
260 Jürgens R Ergeb Inn Med. u. Kinderheilk 53 795 826 1937 Die erblichen Thrombopathien
261 Naunyn Schmiedeberg's Arch. Exptl. Pathol. Pharmakol 222 107 153 1954 Pharmakologische Beeinflussung der Blutgerinnung
262 and H Forsius Schweiz. med. Woch chr 81 1248 1253 1951 Untersuchungen uber bei Konstitutionelle Thrombopathie (v Willebrand Jurgens)
263 Kabashima I and B Suzuki Proc. Imp. Acad., Tokyo 8 494 495 1932 Synthesis of lecithins and cephalins (I) Synthesis of lipalmityl β cephalin and β lecithin

264 Kaesberg P and S Shulman J. Biol. Chem 200 293 296 1953 Electron microscope observations on intermediate polymers in the conversion of fibrinogen to fibrin
265 Kastl O Biochem. Z 274: 452 460 1934 Ist das Thrombin eine Calciumverbindung?
266 Katz S S Shulman I Tinoco Jr I H Billick K Gutfreund and J D Ferry Arch. Biochem. and Biophy. ics 47 165 173 1953 The conversion of fibrinogen to fibrin XIV The effect of calcium on the formation and dissociation of intermediate polymers
267 Kite G L J. Infectious Diseases 15: 319 330 1914 Some structural transformation of the red cells of vertebrates
268 Klein P D and W H Seegers Blood 5 742 752 1950 The nature of plasma antithrombin activity
269 Knisely M H W K Stratman Thomas T S Eliot and E H Bloch J Nat. Malaria Soc 4: 285 300 1945 Knowlesi malaria in monkeys I Microscopic pathological circulatory physiology of rhesus monkeys during acute Plasmodium knowlesi malaria (A motion picture)
270 Koller F Blood 9: .86 290 1954 Is hemophilia a nosologic entity?
271 - A Loeliger and F Duckert Acta Ha matol 6 1 18 1951 Experiments on a new clotting factor
272 Kunitz M J. Gen. Physiol 29 149 154 1946 Crystalline soy bean trypsin inhibitor
273 Laki K Studies Inst. Med. Chem., Univ. Sz ged (Szent Gyorgyi A ed) S Karger Basel 2 27 35 1942 Uber die Fibrinogen Fibrinumwandlung
274 - in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 4 217 225 1951 The transition of fibrinogen to fibrin
275 - Blood 8: 845 856 1953 The clotting of fibrinogen
276 Physiol. Rev 34: 730 735 1954 Chemistry of prothrombin and some of its reactions
277 - and L Lorand Science 108 280 1948 On the solubility of fibrin clots
278 Laki K and W F H M Mommaerts Nature 156 664 1945 Transition of fibrinogen to fibrin as a two step procedure
279 Lampert J and A. Ott Z. ges. Exptl.Med 94 309 322 1934 Blutserumausscheidung und Synärese Der Einfluss physikalischer Faktoren
280 Lamy F and D F Waugh Physiol. Rev 34 722 735 1954 Transformation of prothrombin into thrombin
281 Langdell R D J B Graham and K M Brinkhous Proc. Soc Exptl Biol.Med 74: 424 427 1950 Prothrombin utilization during clotting comparison of results with the two stage and one stage methods
282 Langdell R D R H Wagner and K M Brinkhous J Lab. Clin Med 41: 637 647 1953 Effect of antihemophilic factor on one stage clotting tests A presumptive test for hemophilia and a simple one stage antihemophilic factor assay procedure
283 Lawrence J S and W N Valentine Blood 2 40 49 1947 The blood platelets the rate of their utilization in the cat

284 Leathes J B Lancet (1): 957-962 1925 Rôle of fats in vital phenomena (Part 3 of Groonian Lect)

285 Lee C Y S A Johnson and W H Seegers J. Michigan State Med. Soc 54 801-804 1955 Clotting of blood with Russell's viper venom

286 Lee R I and P D White Am. J. Med. Sci 145: 495 503 1913 A clinical study of the coagulation time of blood

287 Lenggenhager K Weitere Fortschritte in der Blutgerinnungslehre G Thieme Stuttgart 2 ed 1949 243 pp

288 Lepp E Can. J. Med. Technol 11 64 69 1949 Silicone surfaces and blood clotting

289 Le Sourd L and P H Pagniez J. Physiol. et pathol. gén 15 812 825 1913 Le retraction du caillot sanguin et les plaquettes

290 Lewis J H and J H Ferguson J. Clin Invest 27: 778 784 1948 Thrombin formation I The role of calcium serum Ac Globulin and tissue thromboplastin

291 J Clin Invest 29: 486 490 1950 Studies on a proteolytic enzyme system of the blood I Inhibition of fibrinolysin

292 - -N. Carolina Med. J 13: 196 203 1952 Fibrinolytic enzyme system of dog serum

292a - - (Fr transl) Rev. d'Hématol 7: 6 19 1952 Activation de la profibrinolysine

293 - -Am. J. Physiol 170: 636 641 1952 Studies on a proteolytic enzyme system of the blood V Activation of profibrinolysin by trypsin

294 - Proc. Soc. Exptl. Biol. Med 82: 445 448 1953 Hemorrhagic diathesis due to PTC (plasma thromboplastin component) deficiency

295 - - - J. Clin. Invest 32: 915-921 1953 Partial purification of human prothrombin and proconvertin: their characteristics and interaction

296 - - - Am. J. Diseases Children 88 711 714 1954 Afibrinogenemia: report of a case

297 -Blood 10: 351 356 1955 Hypoproaccelerinemia

298 Lewis J H J W Fresh and J H Ferguson Proc. Soc. Exptl Biol. Med 84: 651 654 1953 Congenital hypoproconvertinemia

299 Lewis J H A C Howe and J H Ferguson J. Clin. Invest 28 1507-1510 1949 Thrombin formation II Effects of lysin (fibrinolysin plasmin) on prothrombin Ac globulin and tissue thromboplastin

300 Link T Z. Immunitätsforsch. 85 504 512 1935 Der Einfluss der Schlangengifte auf die Blutgerinnung

301 Loomis E C in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 5: 280 328 1952 Fibrinolysin and antifibrinolysin a proteolytic enzyme system in blood

302 Loomis E C C George Jr and A Ryder Arch. Biochem 12: 1 5 1947 Fibrinolysin: nomenclature unit assay preparation and properties

303 Loomis E C A Ryder and C George Jr Arch. Biochem 20 444 450 1949 Fibrinolysin and antifibrinolysin: biochemical concentration of antifibrinolysin

304 Loránd L Hung. Acta Physiol 1: 192-197 1948 A study on the solubility of fibrin clots in urea

305 Nature 166: 694 1950 Fibrin clots

306 Biochem. J 52: 200 203 1952 Fibrino peptide

307 - Physiol. Rev 34: 742 752 1954 Interaction of thrombin and fibrinogen

308 - and W R Middlebrook Biochem. J 52: 196 199 1952 The action of thrombin on fibrinogen

309 - -Biochim et biophys. Acta 9: 581 582 1952 Studies on fibrino peptide

310 Lovelock J F and B M Porterfield Biochem. J 50 415 420 1951 52 Blood clotting: the function of electrolytes and of calcium

311 Löwit M Sitzber. Akad. Wiss. Wien., Math.-naturw 89: 270-307 1884 Beitrag zur Lehre von der Blutgerinnung

312 Macfarlane R G and R Biggs J. Clin. Pathol 6: 3 8 1953 A thrombin generation test: the application in haemophilia and thrombocytopenia

313 - - Privy Council Med Res Council Mem No 32 The diagnosis and treatment of haemophilia and its related conditions Her Majesty's Stat Office London 1955 pp 1 23

314 - -Le Sang 26 181-190 1955 Thromboplastin generation with particular reference to haemophilia

315 Magalini S I and M Stefanini Proc. Soc. Exptl. Biol. Med 90: 615 617 1955 Failure of synthetic 5 hydroxytryptamine creatinine sulfate to enhance clot retraction of platelet poor human plasma

316 --- Federation Proc 15: 123 124 1956 (Abstr) Retractin: clot retraction promoting factor in platelets and tissues

317 Mann F D Am. J. Clin. Pathol 19: 861 864 1949 Co thromboplastin assay

318 - - Federation Proc 15 524 1956 (Abstr) Complex of prothrombin conversion factors

319 --- N W Barker and M Hurn Am. J. Physiol 164 105-110 1951 Co thromboplastin a probable factor in coagulation of the blood

320 Mann F D M Hurn and T B Magath Proc. Soc. Exptl. Biol Med 66: 33 35 1947 Observations on the conversion of prothrombin to thrombin

321 Mann F D M Hurn and D R Mathieson Am. J. Physiol 158: 84 88 1949 Platelets as foci in the coagulation of blood

322 Mann F D E S Shonyo and F C Mann Am. J. Physiol 164 111 116 1951 Effect of removal of the liver on blood coagulation

323 McDonald M R and M Kunitz J. Gen. Physiol 29: 155-156 1946 An improved method for the crystallization of trypsin

324 McClaughry R I and W H Seegers Blood 5 303-312 1950 Prothrombin thromboplastin Ac globulin and platelet accelerator: quantitative interrelationships

325 McLean F C and A B Hastings J. Biol. Chem 108: 285-322 1935 The state of calcium in the fluids of the body I The conditions affecting the ionization of calcium

326 McLean J Am. J Physiol 43: 586-596 1917 The relation between the thromboplastic action of cephalin and its degree of unsaturation

327 Mellanby J J. Physiol 38: 28-112; 441-503 1909 The coagulation of blood

328 - - Proc. Roy. Soc (London) B 107: 271 285 1930 Prothrombase its preparation and properties

329 - -- Proc. Roy. Soc (London) B 113: 93-106 1933 Thrombase Its preparation and properties

330 - -Proc. Roy. Soc (London) B 116: 1 9 1934 Heparin and blood coagulation

331 Mertz E T and C A Owen Proc. Soc. Exptl. Biol. Med 43: 204 205 1940 Imidazole buffer: its use in blood clotting studies

332 Mertz E T W H Seegers and H P Smith Proc. Soc. Exptl Biol. Med 42: 604 605 1939 Prothrombin thromboplastin and thrombin: quantitative interrelationships

333 Mills C A Am J. Physiol 76: 632 641 1926 Blood clotting studies in hemophilia

334 - - - Am. J. Med. Sci 172: 501 511 1926 Considerations of the problem of blood clotting

335 -Chinese J Physiol 1: 235 244 1927 The role of platelets in blood clotting

336 Chinese J. Physiol 1: 435 438 1927 Is cephalin necessary in the activation of prothrombin?

337 - - J. Orient. Med 8: 58 62; 85 89; 105 114; 9: 1-10 1928 Blood coagulation and the control of hemorrhage

338 - -- -Am. J.Physiol 95 1-6 1930 Do blood platelets plasma and tissues yield thrombin or tissue fibrinogen?

339 Milstone J H J. Gen. Physiol 31: 301 324 1948 Three stage analysis of blood coagulation

340 -- Proc. Soc. Exptl. Biol. Med 68: 225-228 1948 Activation of prothrombin by platelets plus globulin

341 Minot G R and R I Lee Arch. Internal Med 18 474-495 1916 The blood platelets in hemophilia

342 Monkhouse F C and L B Jaques J. Lab. Clin. Med 36: 782 789 1950 An improved method for the extraction of heparin from blood

343 Moolton S E and L Vroman Am. J Clin. Pathol 19: 701-709 1949 Adhesiveness of blood platelets in thromboembolism and hemorrhagic disorders I Measurement of platelet adhesiveness by the glass wool filter

344 Morawitz P Deut. Arch. f. klin. Med 79: 1 28 1904 Beitrag zur Kenntniss der Blutgerinnung I Mitteilung

345 -- Hofmeister's Beitr. chem. Physiol. u. Pathol 5 133 141 1904 Beitrag zur Kenntniss der Blutgerinnung

346 - Ergeb. Physiol 4 307 422 1905 Die Chemie der Blutgerinnung

347 - - -in Handbuch der Biochemie des Menschen und der Tiere C Oppenheimer ed Gustav Fischer Jena 2 ed 4 44 77 1925 Blutgerinnung

348 Morrison P R J. Am. Chem. Soc 69: 2723 2731 1947 Preparation and properties of serum and plasma proteins IV Some factors influencing the quantitative determination of fibrinogen

349 Murphy R C and W H Seegers Am. J. Physiol 154: 134 139 1948 Concentration of prothrombin and Ac globulin in various species

350 Naeye R L Proc. Soc. Exptl. Biol. Med 91: 101 104 1956 Plasma thromboplastin component: influence of coumarin compounds and vitamin K on its activity in serum

351 Nolf P Medicine. 17: 381 411 1938 The coagulation of the blood

352 Nordbo R Skand. Arch. Physiol 75: Suppl 11 1 46 1936 Uber die Aktivitatskoeffizienten der Kalziumionen und der Oxalationen in Plasma und uber die Bedeutung der Kalziumionenkonzentration für die Gerinnung des Blutes

353 Northrop J H M Kunitz and R M Herriott Crystalline enzymes Columbia Univ Press New York 2nd ed 1948 352 pp

354 O'Brien J R J. Clin. Pathol 9: 47 51 1956 The similarity of the action of phosphatidyl ethanolamine and platelets in blood coagulation

355 Oliver W W Science 40: 645 648 1914 The crenation and flagellation of human erythrocytes

356 Opitz H and G Matzdorff Deut. Med. Wochschr 47: 504 1921 Eine Fehlerquelle bei der Bestimmung der Retraktilitat des Blutkuchens

357 Osler W Proc. Roy. Soc (London) 22: 391 393 1873 74 An account of certain organisms occurring in the liquor sanguinis

358 Overman R S In Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 1: 144 177 1948 A lipid inhibitor from beef brain rabbit lung soy bean phosphatides and normal human plasma

359 in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2 29 50 1949 The chemical purification and mode of action of a thromboplastic inhibitor

360 Owren P A Bull. schweiz Akad. Med. Wiss 3: 163 176 1947 New factors concerned in coagulation of blood

361 -Acta Med. Scand Suppl 194: 1 327 1947 The coagulation of blood Investigations on a new clotting factor

362 Lancet (1): 446 448 1947 Parahaemophilia haemorrhagic diathesis due to absence of a previously unknown clotting factor

363 in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 5: 92 170 1952 New clotting factors

364 Am. J. Med 14: 201 215 1953 Prothrombin and accessory factors

365 - and K Aas Scand. J. Clin. Lab. Invest 3: 201 208 1951 The control of dicumarol therapy and the quantitative determination of prothrombin and proconvertin

366 Owren P A and C J Bjerkelund Scand J. Clin. Lab. Invest 1 162-163 1949 A new previously unknown clotting factor
367 Page I H Physiol. Rev 34: 563 588 1954 Serotonin (5-Hydroxytryptamine)
368 Patek A J and R P Stetson J. Clin Invest 15: 531-542 1936 Hemophilia I The abnormal coagulation of the blood and its relation to the blood platelets
369 Patton T B A G Ware and W H Seegers Blood 3: 656 659 1948 Clotting of plasma and silicone surfaces
370 Pavlovsky A Blood 2 185-191 1947 Contribution to the pathogenesis of hemophilia
371 Pedersen K O Ultra centrifugal studies on serum and serum fractions Almquist & Wiksells AB Uppsala 1945 178 pp (pp 52-57)
372 Pekelharing C A Internat. Beitr. z. Wissensch. Med (Festschr R Virchow) 1 433-456 1891 Über die Bedeutung der Kalzsalze für die Gerinnung des Blutes
373 - Verhandel. d. Koninkl. Akad. Wetenschap Amsterdam Afdeel Natuurk Sect 2 Pt 1 pp 1 52 1892 Untersuchungen uber das Fibrinferment
374 Penick G D E P Cronkite I D Godwin and K M Brinkhous Proc. Soc. Exptl. Biol. Med 78 732-734 1951 Plasma anti hemophilic activity following total body irradiation
375 Pickering J W The blood plasma in health and disease Heinemann London 1928 203 pp
376 Pinniger J L and F T G Prunty Brit. J. Exptl Pathol 27 200 210 1946 Some observations on the blood clotting mechanism The role of fibrinogen and platelets with reference to a case of congenital afibrinogenemia
377 Pitney W R and J V Dacie J. Clin. Pathol 6: 9 14 1953 A simple method of studying the generation of thrombin in recalcified plasma
378 - - -- Brit. Med. Bull 11 11-15 1955 Haemophilia and allied disorders of blood coagulation
379 Pope C G Brit. J. Exptl. Pathol 19: 245 251 1938 Disaggregation of proteins by enzymes
380 Presnell A K Am. J. Physiol 122: 596-601 1938 Thrombin a proteolytic fibrinogenase
381 Quick A J J Immunol 29: 87 97 1935 On the relationship between complement and prothrombin
382 - Am. J. Physiol 123 712 719 1938 The normal antithrombin of the blood and its relation to heparin
383 The hemorrhagic diseases and the physiology of hemostasis C C Thomas Springfield 1942 340 pp
384 - --Am. J. Physiol 140: 212 220 1943 On the constitution of prothrombin
385 Am. J. Med. Sci 214 272 280 1947 Studies on the enigma of the hemostatic dysfunction of hemophilia
386 - Am. J. Clin. Pathol 19: 1016 1023 1949 The coagulation mechanism with specific reference to the interpretation of prothrombin time and a consideration of the prothrombin consumption test
387 -- - The physiology and pathology of hemostasis Lea & Febiger Philadelphia 1951 188 pp

388 Quick A J and J E Favre Gilly Blood 4: 1281 1289 1949 The prothrombin consumption test: its clinical and theoretic implications
389 Quick A. J and C V Hussey Brit. Med. J (1): 934 937 1955 Prothrombin and the one-stage prothrombin time
390 Quick A J A. V Pisciotta and C V Hussey Arch. Internal Med 95: 2 14 1955 Congenital hypoprothrombinemic states
391 Quick A J J N Shanberge and M Stefanini Am. J. Med. Sci 217: 198 205 1949 The role of platelets in the coagulation of the blood
392 - - J. Lab. Clin. Med 34: 761-767 1949 The coagulation defect in thrombocytopenic purpura
393 Quick A J M Stanley Brown and F W Bancroft Am. J. Med Sci 190 501 511 1935 A study of the coagulation defect in hemophilia and in jaundice
394 Quick A J and M Stefanini J. Gen. Physiol 32: 191 202 1948 The chemical state of the calcium reacting in the coagulation of blood
395 - J. Lab. Clin. Med 33 819 826 1948 The concentration of the labile factor of the prothrombin complex in human dog and rabbit blood: its significance in the determination of prothrombin activity
396 - J. Lab. Clin. Med 34 1203 1215 1949 The state of component A (prothrombin) in human blood evidence that it is partly free and partly in an inactive or precursor form
397 Ranvier M Compt. rend. soc. biol 5: 46 51 1873 Du mode de formation de la fibrine dans le sang extrait des vaisseax
398 Rapoport S Proc. Soc. Exptl. Biol. Med 64: 478 486 1947 New formula for dilution curve of plasma prothrombin Normal standards and changes in pathological conditions
399 Rapport M M J. Biol. Chem 180 961 969 1949 Serum vaso constrictor (serotonin) Presence of creatinine in complex Proposed structure of vasoconstrictor principle
400 - A. A. Green and I H Page Science 108 329 330 1948 Crystalline serotonin

401 Ratnoff O D and J E Colopy J. Clin. Invest 34 602 613 1955 A familial hemorrhagic trait associated with a deficiency of a clot promoting fraction of plasma
402 Ratnoff O C R C Hartmann and C L Conley J. Exptl. Med 91 123 133 1950 Studies on a proteolytic enzyme in human plasma V The relationship between the proteolytic activity of plasma and blood coagulation
403 Riley J F and G B West J. Pathol. Bacteriol 69 269 282 1955 Tissue mast cells studies with a histamine liberator of low toxicity (compound 48/80)
404 Ringer S and J Sainsbury J. Physiol 11 369-383 1890 The influence of certain salts upon the act of clotting
405 Robbins F C Am. J. Phy iol 142 581 588 1944 Study on conversion of fibrinogen to fibrin
406 Robinson D S and J C F Poole Quart. J. Exptl. Physiol 41 36 50 1956 The similar effect of chylomicra and ethanol amine phosphatide on the generation of thrombin during coagulation

407 Rocha e Silva M and R M Teixeira Proc. Soc. Exptl. Biol. Med 61 376 382 1946 Role played by leucocytes platelets and plasma trypsin in peptone shock in the dog

408 Rolleston H Proc. Roy. Soc. Med 27 31 48 1934 The history of haematology

409 Rosenfeld G and B Jansky Science 116 36 37 1952 The accelerating effect of calcium on the fibrinogen fibrin transformation

410 Rosenfield R E and H S Tuft Am. J. Clin Pathol 17 405 412 1947 Estimation of prothrombin level from prothrombin time

411 Rosenthal N in Handbook of hematology H Downey ed Paul B Hoeber New York 1 Sect VII 447 496 1938 Blood platelets and megakaryocytes

412 Rosenthal R L Am. J Med 17 57 69 1954 Hemophilia and hemophilia like diseases caused by deficiencies in plasma thromboplastin factors antihemophilic globulin (AHG) plasma thromboplastin component (PTC) and plasma thromboplastin antecedent (PTA)

413 O H Dreskin and N Rosenthal Proc. Soc. Exptl. Biol Med 82 171 174 1953 New hemophilia like disease caused by deficiency of a third thromboplastic factor

414 Blood 10 120 131 1955 Plasma thromboplastin antecedent (PTA) deficiency clinical coagulation therapeutic and hereditary aspects of a new hemophilia like disease

415 Roskam J Physiologie normale et pathologique du globulin Presses Univ de France Paris 19-7 151 pp

416 Rumpf F Biochem. Z 55 101 115 1913 Ueber den Einfluss der Lipoide auf die Gerinnung des Blutes

417 Sabbatini L Arch. ital. de biol 36 397 415 416 418 1901 39 333 375 1903
(a) Calcium et citrate trisodique dans la coagulation du sang de la lymph et du lait
(b) Fonction biologique du calcium I Action antagoniste entre le citrate trisodique et le calcium
(c) II Le calcium dans la coagulation du sang

418 Sahli H Z. klin. Med 56 264 315 1904 05 Ueber das Wesen der Hamophilie

419 Samuels P B and D R Webster Ann. Surg 136 422 428 1952 The role of venous endothelium in the inception of thrombosis

420 Schmidt A Zur Blutlehre F C W Vogel Leipzig 1892 270 pp

42 Weitere Beitrage zur Blutlehre J F Bergmann Wiesbaden 1895 250 pp

422 Schmitt F O Harvey Lectures 40 249 268 1945 Ultrastructure and the problem of cellular organization

423 Schultze H E and G Schwick Hoppe Seyler s Z. physiol. Chem 289 26 43 1951 Über den Mechanismus der Thrombinbildung im isolierten System

424 Seegers W H J. Biol. Chem 136 103 111 1940 Purification of prothrombin and thrombin chemical properties of purified preparations

425 - Harvey Lectures 47 180-220 1951 52 Coagulation of the blood

426 Seegers W H National Research Council Panel on Blood Coagulation Washington D C April 1955 (Mimeo) A contemporary view of platelets as they relate to the blood clotting mechanisms

427 T Abe and R L Fenichel Can. J. Biochem. Physiol 34: 270 272 1956 Electrophoresis of autoprothrombin and prothrombin

428 Seegers W H K M Brinkhous H P Smith and E D Warner J. Biol. Chem 126: 91 95 1938 The purification of thrombin

429 Seegers W H S A Johnson and N Alkjaersig Federation Proc 15: 351 352 1956 (Abstr) Interrelationships between prothrombin and certain derivatives of prothrombin

430 Seegers W H and E C Loomis Science 104 461 462 1946 Prothrombin and fibrinolysin

431 and J M Vandenbelt Arch. Biochem 6: 85 95 1945 Preparation of prothrombin products isolation of prothrombin and its properties

432 Seegers W H R I McClaughry and J L Fahey Blood 5: 421 433 1950 Some properties of purified prothrombin and its activation with citrate

433 Seegers W H and D A McGinty J. Biol. Chem 146: 511 518 1942 Further purification of thrombin: probable purity of products

433a Seegers W H K D Miller E B Andrews and R C Murphy Am. J. Physiol 169: 700 711 1952 Fundamental interactions and effect of storage ether adsorbants and blood clotting on plasma antithrombin activity

434 Seegers W H M Nieft and E C Loomis Science 101 520 521 1945 Note on the adsorption of thrombin on fibrin

435 Seegers W H H P Smith E D Warner and K M Brinkhous J. Biol. Chem 123: 751 754 1938 The purification of prothrombin

436 Shapiro S B Sherwin M Redish and H A Campbell Proc. Soc Exptl. Biol. Med 50: 85 89 1942 Prothrombin estimation: a procedure and clinical interpretations

437 Sherry S W Troll and H Glueck Physiol. Rev 34: 736 741 1954 Thrombin as a proteolytic enzyme

438 Shulman S Arch. Biochem 30 353 371 1951 The conversion of fibrinogen to fibrin IV Reversible inhibition of the reaction

439 Nature 171 606 607 1953 The fibrin serum factor

440 S Katz and J D Ferry J. Gen. Physiol 36 759 766 1953 The conversion of fibrinogen to fibrin XIII Dissolution of fibrin and inhibition of clotting by various neutral salts

441 Siedentopf H and R Zsigmondy Ann. Physik 10 1 39 1903 Ueber Sichtbarmachung und Grossenbestimmung ultramikroscopischen Teilchen u s w

442 Smith H P E D Warner and K M Brinkhous Am. J. Physiol 107: 63 69 1934 Lung extract and blood clotting

443 J. Exptl. Med 66 801 811 1937 Prothrombin deficiency and the bleeding tendency in liver injury (chloroform intoxication)

407 Rocha e Silva M and R M Teixeira Proc. Soc Exptl. Biol. Med 61 376 382 1946 Role played by leucocytes platelets and plasma trypsin in peptone shock in the dog

408 Rolleston H Proc. Roy. Soc Med 27 31 48 1934 The history of haematology

409 Rosenfeld G and B Jansky Science 116 36 37 1952 The ac celerating effect of calcium on the fibrinogen fibrin transformation

410 Rosenfield R E and H S Tuft Am. J. Clin. Pathol 17 405 412 1947 Estimation of prothrombin level from pro thrombin time

411 Rosenthal N in Handbook of hematology H Downey ed Paul B Hoeber New York 1 Sect VII 447 496 1938 Blood platelets and megakaryocytes

412 Rosenthal R L Am. J Med 17 57 69 1954 Hemophilia and hemophilia like diseases caused by deficiencies in plasma thromboplastin factors antihemophilic globulin (AHG) plasma thromboplastin component (PTC) and plasma thromboplastin antecedent (PTA)

413 O H Dreskin and N Rosenthal Proc. Soc. Exptl. Biol Med 82 171 174 1953 New hemophilia like disease caused by deficiency of a third thromboplastic factor

414 Blood 10 120 131 1955 Plasma thromboplastin antece dent (PTA) deficiency clinical coagulation therapeutic and hereditary aspects of a new hemophilia like disease

415 Roskam J Physiologie normale et pathologique du globulin Presses Univ de France Paris 1927 151 pp

416 Rumpf F Biochem. Z 55 101 115 1913 Ueber den Einfluss der Lipoide auf die Gerinnung des Blutes

417 Sabbatini L Arch. ital. de biol 36 397 415; 416 418 1901; 39 333 375 1903
(a) Calcium et citrate trisodique dans la coagulation du sang de la lymph et du lait
(b) Fonction biologique du calcium I Action antagoniste entre le citrate trisodique et le calcium
(c) II Le calcium dans la coagulation du sang

418 Sahli H Z. klin. Med 56 264 315 1904 05 Ueber das Wesen der Hamophilie

419 Samuels P B and D R Webster Ann. Surg 136 422 428 1952 The role of venous endothelium in the inception of thrombosis

420 Schmidt A Zur Blutlehre F C W Vogel Leipzig 1892 270 pp

42 Weitere Beitrage zur Blutlehre J F Bergmann Wiesbaden 1895 250 pp

422 Schmitt F O Harvey Lectures 40 249 268 1945 Ultrastruc ture and the problem of cellular organization

423 Schultze H E and G Schwick Hoppe Seyler's Z. physiol. Chem 289 26 43 1951 Über den Mechanismus der Thrombin bildung im isolierten System

424 Seegers W H J. Biol. Chem 136 103 111 1940 Purification of prothrombin and thrombin chemical properties of purified preparations

425 -- Harvey Lectures 47 180-220 1951 52 Coagulation of the blood

461 Sykes E M Jr W H Seegers and A G Ware Proc. Soc Exptl. Biol. Med 67: 506 507 1948 Effect of acute liver damage on Ac globulin activity of plasma

462 Tagnon H J J. Lab. Clin. Med 27: 1119 1131 1942 The significance of fibrinolysis in mechanism of coagulation of blood

463 Tanturi C A and R F Banfi J. Lab. Clin. Med 31 703 713 1946 An improved method for the determination of plasma prothrombin

464 Thudichum J L W A manual of chemical physiology; its points of contact with physiology Wm Wood & Co New York 1872 195 pp

465 A treatise on the chemical constitution of the brain Bailliere Tindall & Cox London 1884 262 pp

466 Tinoco I Jr and J D Ferry Arch. Biochem. and Biophys 48 7 16 1954 The conversion of fibrinogen into fibrin XV Sedimentation studies of the polymerization of fibrinogen at high pH

467 Tocantins L M Am. J. Physiol 110: 278 286 1934 Platelets and the spontaneous syneresis of blood clots

468 Am. J. Physiol 114: 709 715 1936 Platelets and the structure and physical properties of blood clots

469 Medicine 17 155 258 1938 The mammalian blood platelet in health and disease

470 Am. J. Physiol 139 265 279 1943 Demonstration of antithromboplastin activity in normal and hemophilic plasmas

471 in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 5: 247 279 1952 Antithromboplastin activity of the plasma of animals exposed to ionizing radiations

472 Blood 9 281 285 1954 Hemophilic syndromes and hemophilia

473 (ed) The coagulation of blood; methods of study Grune & Stratton New York 1955 240 pp

474 and R T Carroll in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2 11 27 1949 Separation and assay of a lipid antithromboplastin from human brain blood plasma and plasma fractions

475 and R H Holburn Blood 6 720 739 1951 The clot accelerating effect of dilution of blood and plasma Relation to the mechanism of blood coagulation in normal and hemophilic blood

476 Travis B L and J H Ferguson J. Clin. Invest 30 112 123 1951 Proteolytic enzymes and platelets in relation to blood coagulation

477 Trum B F and J H Rust Proc. Soc. Exptl. Biol. Med 82 347 351 1953 Whole blood clotting clot retraction and prothrombin utilization in burros following total body gamma radiation

478 Tullis J L New Engl. Med. J 249 591 595 1953 Platelet antibody tests in diagnosis of purpura

444 Soulier J P Le Sang 19: 78-94 1948 Nouvelle méthode de diagnostic de l'hemophilie; utilisant les sangs veineux e' capillaires coagules Comparaison entre les resultats obtenus dans l'hemophilie et dans autres syndromes hemorragiques

445 - - -and M J Larrieu Rev. Hematol 9: 77 122 1954 Syndrome de Willebrand Jürgens et thrombopathies; etude de 65 cas; essai de classification

446 Spaet T P M Aggeler and B G Kinsell J. Clin. Invest 33: 1095 1102 1942 A possible fourth thromboplastic component

447 Spagnol G Rev. sud amer. endocrinol., immunol., quimioterap 17: 619 633 1934 Cefalinas y coagulabilidad sanguinea

448 Speer R J J M Hill M Maloney and A Roberts J Lab. Clin Med 45: 730 739 1955 Hemorrhagic diathesis associated with hyperheparinemia

449 Stefanini M Am. J. Med 14: 64 86 1953 Mechanism of blood coagulation in normal and pathological conditions

450 and I S Murphy J. Clin. Invest 35 355 361 1956 Studies on platelets human platelets as source of antifibrinolysin

451 Stefanini M and J B Chatterjea Proc. Soc. Exptl. Biol. Med 79 623 629 1952 Studies on platelets IV A thrombo cytopenic factor in normal human plasma or serum

452 Stefanini M and W H Crosby Blood 5: 964 973 1950 The one stage prothrombin consumption test: clinical value in the identification of thromboplastin deficiency disease

453 Stefanini M and W Dameshek The hemorrhagic disorders, a clinical and therapeutic approach Grune & Stratton New York 1955 368 pp

454 - J B Chatterjea E Adelson and I B Mednicoff Blood 8 26 64 1953 Studies on platelets IX Observations on the properties and mechanism of action of a potent platelet agglutinin detected in the serum of a patient with idiopathic thrombocytopenic purpura (with a note on the pathogenesis of the disease)

455 Stefanini M G I Plitman W Dameshek J B Chatterjea and I B Mednicoff J. Lab. Clin. Med 42 723 738 1953 Studies on platelets XI Antigenicity of platelets and evidence for platelet groups and types in man

456 Sternberger L A Brit. J. Exptl. Pathol 28 168 177 1947 The stabilization of thrombin in plasma development of a simple two stage method for the determination of prothrombin

457 ---J. Am. Med. Assoc 150: 1591 1593 1952 Preliminary clinical evaluation of thrombin recovery test

458 Strughold H and E Wöhlisch Hoppe Seyler's Z. physiol. Chem 223 267 280 1934 Ist das Thrombin ein proteolytisches Ferment?

459 Stübel H Pfluger's Arch. ges. Physiol 156 361-400 1914 Ul tramikroskopisch Studien uber Blutgerinnung und Thrombocyten

460 Surgenor D M Sci. American 190: 54 62 1954 Blood

461 Sykes E M Jr W H Seegers and A G Ware Proc. Soc Exptl. Biol. Med 67: 506 507 1948 Effect of acute liver damage on Ac globulin activity of plasma
462 Tagnon H J J. Lab. Clin. Med 27: 1119 1131 1942 The significance of fibrinolysis in mechanism of coagulation of blood
463 Tanturi C A and R F Banfi J. Lab. Clin. Med 31: 703 713 1946 An improved method for the determination of plasma prothrombin
464 Thudichum J L W A manual of chemical physiology: its points of contact with physiology Wm Wood & Co New York 1872 195 pp
465 A treatise on the chemical constitution of the brain Baillière Tindall & Cox London 1884 262 pp
466 Tinoco I Jr and J D Ferry Arch. Biochem. and Biophys 48 7 16 1954 The conversion of fibrinogen into fibrin XV Sedimentation studies of the polymerization of fibrinogen at high pH
467 Tocantins L M Am. J. Physiol 110 278 286 1934 Platelets and the spontaneous syneresis of blood clots
468 Am. J. Physiol 114: 709 715 1936 Platelets and the structure and physical properties of blood clots
469 Medicine 17 155 258 1938 The mammalian blood platelet in health and disease
470 - Am. J. Physiol 139 265 279 1943 Demonstration of antithromboplastin activity in normal and hemophilic plasmas
471 - - in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 5 247 279 1952 Antithromboplastin activity of the plasma of animals exposed to ionizing radiations
472 - Blood 9: 281 285 1954 Hemophilic syndromes and hemophilia
473 (ed) The coagulation of blood, methods of study Grune & Stratton New York 1955 240 pp
474 -- and R T Carroll in Josiah Macy Jr Found Confs on Blood clotting and allied problems J E Flynn ed 2 11 27 1949 Separation and assay of a lipid antithromboplastin from human brain blood plasma and plasma fractions
475 - and R H Holburn Blood 6 720 739 1951 The clot accelerating effect of dilution of blood and plasma Relation to the mechanism of blood coagulation in normal and hemophilic blood
476 Travis B L and J H Ferguson J. Clin. Invest 30: 112 123 1951 Proteolytic enzymes and platelets in relation to blood coagulation
477 Trum B F and J H Rust Proc. Soc. Exptl. Biol. Med 82 347 351 1953 Whole blood clotting clot retraction and prothrombin utilization in burros following total body gamma radiation
478 Tullis J L New Engl. Med. J 249 591 595 1953 Platelet antibody tests in diagnosis of purpura

479 Tullis J L (ed) Blood cells and plasma proteins, their state in nature Academic Press New York 1953 436 pp Chap 5 pp 143 155 The platelets of human blood their morphology interactions and preservation
480 Tyson T L and R West Proc. Soc. Exptl. Biol. Med 36 494 496 1937 Effect of trypsin on the clotting of the blood in hemophilia
481 Van Creveld S and M M P Paulssen Lancet (1) 23 25 1952 Isolation and properties of the third clotting factor in blood platelets
482 Virchow R Z. f. rat. Med 4 262 292 1846 Ueber die chemische Eigenschaften des Faserstoffs
483 Virchow's Arch pathol. Anat. u Physiol 6 562 572 1853 Ueber das ausgebreitete Vorkommen einer dem Nervenmark analogen Substanz in den theirischen Geweben
484 Von Willebrand E A Finska Lak. sallsk. Handl. Helsingfors 68 87 92 1926 Hereditare Pseudohemofili
485 Acta Med. Scand 76 521 550 1931 Ueber hereditare Pseudohamophilie
486 and R Jurgens Klin. Wochschr 12 414 417 1933 Ueber eine neue Bluterkrankheit die konstitutionelle Thrombopathie
487 Wagner R H W M Brannan and K M Brinkhous Proc. Soc Exptl. Biol. Med 89 266 270 1955 Antiaccelerator (anticonvertin) activity of canine plasma and serum
488 Wagner R H J G McAllister III and K M Brinkhous Federation Proc 15 537 1956 (Abstr) Selective adsorption of antihemophilic factor (AHF) and fibrinogen from canine plasma
489 Waksman S A Am. J. Physiol 46 375 395 1918 Studies on the thromboplastic action of cephalin
490 Ware A G J L Fahey and W H Seegers Am. J. Physiol 154 140 147 1948 Platelet extracts fibrin formation and interaction of purified prothrombin and thromboplastin
491 Ware A G and G F Lanchantin Physiol. Rev 34 714 721 1954 Purification of fibrinogen prothrombin and thrombin
492 Ware A G and W H Seegers Am. J. Physiol 152 567 576 1948 Serum Ac globulin formation from plasma Ac globulin role in blood coagulation partial purification properties and quantitative determination
493 J Biol Chem 172 699 705 1948 Plasma accelerator globulin partial purification quantitative determination and properties
494 J Biol Chem 174 565 575 1948 Studies on prothrombin purification inactivation with thrombin and activation with thromboplastin and calcium
495 - Am. J. Clin. Pathol 19 471 482 1949 Two-stage procedure for the quantitative determination of prothrombin concentration
496 Warner E D K M Brinkhous and H P Smith Am. J. Physiol 114 667 675 1936 A quantitative study of blood clotting: prothrombin fluctuations under experimental conditions
497 - - Proc. Soc. Exptl. Biol. Med 40 197 200 1939 The prothrombin conversion rate in various species

498 Waters E T J Markowitz and L B Jaques Science 87: 582 583 1938 Anaphylaxis in the liverless dog and observations on the anticoagulant of anaphylactic shock

499 Weitnauer J and E Wöhlisch Biochem. Z 288: 137 144 1936 Die Entbehrlichkeit des Calciums bei der Einwirkung des Thrombin auf das Fibrinogen

500 Wenckert A and I M Nilsson Scand. J. Clin. Lab. Invest 7 Suppl 15 1 97 1955 Thromboplastin and Russell viper venom Investigation of the activation of prothrombin

501 Wilander O Skand. Arch. Physiol 81: Suppl 15 1 89 1938 Studien über Heparin

502 Wilson S J G Eisemann and J H Chance Proc. Soc. Exptl. Biol. Med 81: 317-319 1952 Plasma thrombocytopenic factor as measured by direct and indirect platelet methods

503 Wintrobe M M Clinical hematology Lea & Febiger Philadelphia 3rd ed 1951 1048 pp

504 Wöhlisch E Klin Wochschr 2: 1801 1802 1923 Untersuchungen zur Theorie der Thrombinwirkung

505 Ergeb. Physiol 28: 443 624 1929 Die Physiologie und Pathologie der Blutgerinnung

506 Ergeb. Physiol 43: 174 370 1940 Forstschritte in der Physiologie der Blutgerinnung

507 Wolpers C and H Ruska Klin. Wochschr 18: 1077 1081; 1111 1117 1939 Strukturuntersuchungen zur Blutgerinnung

508 Woolridge L C On the chemistry of the blood, and other scientific papers (Arranged by V Horsely and E Starling) Kegan Paul Trench Trubner & Co London 1893 354 pp

509 Wright H P in Josiah Macy Jr Found Confs on Blood Clotting and allied problems J E Flynn ed 4 119 142 1951 Characteristics of blood platelets Their significance in thrombus formation

510 Wright J H J. Morphol 21: 263 279 1910 The histogenesis of the blood platelets

511 and G R Minot J. Exptl. Med 26 395 410 1917 The viscous metamorphosis of the blood platelets

512 Wycoff H D J. Lab. Clin. Med 47 645 648 1956 A micro assay for plasma fibrinogen

513 Zahn F W Centralbl. f. d. med. Wissensch 10 129 1872 Untersuchungen über Thrombose Bildung der Thrombin

514 Zak E Naunyn Schmiedeberg s Arch. exptl. Pathol. Pharmakol 70 27 54 1912 Studien zur Blutgerinnungslehre

515 Zatti P Boll. soc. ital. biol. sper 24: 22 23 1948 Sulle cause dell' agglutinazione delle piastrine nel sangue in via di coagulazione

516 Zimmermann G Magazin f. d. ges. Heilk 66 3 46 1846 Ueber den rothen Bodensatz den das Blut zuweilen nach der Gerinnung zeigt

517 Virchow s Arch f. Pathol. Anat. u. Physiol 18: 221 242 1860 Zur Blutkorperchenfrage

518 Zucker M B in Josiah Macy Jr Found Confs on Blood Clotting and allied problems J E Flynn ed 4: 143 156 1951 Morphological and physiological studies of platelets and hemostasis

479 Tullis J L (ed) Blood cells and plasma proteins: their state in nature Academic Press New York 1953 436 pp Chap 5 pp 143 155 The platelets of human blood their mor phology interactions and preservation
480 Tyson T L and R West Proc. Soc. Exptl. Biol. Med 36 494 496 1937 Effect of trypsin on the clotting of the blood in hemophilia
481 Van Creveld S and M M P Paulssen Lancet (1) 23 25 1952 Isolation and properties of the third clotting factor in blood platelets
482 Virchow R Z. f. rat Med 4 262 292 1846 Ueber die chemische Eigenschaften des Faserstoffs
483 Virchow's Arch pathol Anat. u. Physiol 6 562 572 1853 Ueber das ausgebreitete Vorkommen einer dem Nervenmark analogen Substanz in den theirischen Geweben
484 Von Willebrand E A Finska Lak. sallsk. Handl. Helsingfors 68 87 92 1926 Hereditare Pseudohemofili
485 - Acta Med. Scand 76 521 550 1931 Ueber hereditare Pseudohamophilie
486 and R Jürgens Klin. Wochschr 12 414 417 1933 Ueber eine neue Bluterkrankheit die konstitutionelle Thrombopathie
487 Wagner R H W M Brannan and K M Brinkhous Proc Soc Exptl. Biol. Med 89 266 270 1955 Antiaccelerator *(anticonvertin) activity of canine plasma and serum*
488 Wagner R H J G McAllister III and K M Brinkhous Federa tion Proc 15 537 1956 (Abstr) Selective adsorption of antihemophilic factor (AHF) and fibrinogen from canine plasma
489 Waksman S A Am. J Physiol 46 375-395 1918 Studies on the thromboplastic action of cephalin
490 Ware A G J L Fahey and W H Seegers Am. J Physiol 154 140 147 1948 Platelet extracts fibrin formation and interaction of purified prothrombin and thromboplastin
491 Ware A G and G F Lanchantin Physiol. Rev 34 714 721 1954 Purification of fibrinogen prothrombin and thrombin
492 Ware A G and W H Seegers Am. J Physiol 152 567 576 1948 Serum Ac globulin formation from plasma Ac globulin role in blood coagulation partial purification properties and quantitative determination
493 J Biol Chem 172 699 705 1948 Plasma accelerator globulin partial purification quantitative determination and properties
494 - J. Biol. Chem 174 565 575 1948 Studies on prothrom bin purification inactivation with thrombin and activation with thromboplastin and calcium
495 - - Am. J. Clin. Pathol 19 471 482 1949 Two stage pro cedure for the quantitative determination of prothrombin concentration
496 Warner E D K M Brinkhous and H P Smith Am. J. Physiol 114: 667 675 1936 A quantitative study of blood clotting: prothrombin fluctuations under experimental conditions
497 - Proc. Soc. Exptl. Biol. Med 40 197 200 1939 The prothrombin conversion rate in various species

498 Waters E T J Markowitz and L B Jaques Science 87: 582 583 1938 Anaphylaxis in the liverless dog and observations on the anticoagulant of anaphylactic shock

499 Weitnauer J and E Wöhlisch Biochem. Z 288: 137 144 1936 Die Entbehrlichkeit des Calciums bei der Einwirkung des Thrombin auf das Fibrinogen

500 Wenckert A and I M Nilsson Scand. J. Clin. Lab. Invest 7 Suppl 15 1 97 1955 Thromboplastin and Russell viper venom Investigation of the activation of prothrombin

501 Wilander O Skand. Arch. Physiol 81: Suppl 15 1 89 1938 Studien über Heparin

502 Wilson S J G Eisemann and J H Chance Proc. Soc. Exptl. Biol. Med 81: 317 319 1952 Plasma thrombocytopenic factor as measured by direct and indirect platelet methods

503 Wintrobe M M Clinical hematology Lea & Febiger Philadelphia 3rd ed 1951 1048 pp

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506 Ergeb. Physiol 43: 174 370 1940 Forstschritte in der Physiologie der Blutgerinnung

507 Wolpers C and H Ruska Klin. Wochschr 18: 1077 1081 1111 1117 1939 Strukturuntersuchungen zur Blutgerinnung

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509 Wright H P in Josiah Macy Jr Found Confs on Blood Clotting and allied problems J E Flynn ed 4: 119 142 1951 Characteristics of blood platelets Their significance in thrombus formation

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511 and G R Minot J. Exptl. Med 26: 395 410 1917 The viscous metamorphosis of the blood platelets

512 Wycoff H D J. Lab. Clin. Med 47: 645 648 1956 A micro assay for plasma fibrinogen

513 Zahn F W Centralbl. f. d. med. Wissensch 10 129 1872 Untersuchungen über Thrombose Bildung der Thrombin

514 Zak E Naunyn Schmi deb rg s Arch. exptl. Pathol. Pharmakol 70 27 54 1912 Studien zur Blutgerinnungslehre

515 Zatti P Boll. soc. ital. biol. sper 24 22 23 1948 Sulle cause dell' agglutinazione delle piastrine nel sangue in via di coagulazione

516 Zimmermann G Magazin f. d. ges. Heilk 66 3 46 1846 Ueber den rothen Bodensatz den das Blut zuweilen nach der Gerinnung zeigt

517 - Virchow s Arch. f. Pathol. Anat. u. Physiol 18 221 242 1860 Zur Blutkorperchenfrage

518 Zucker M B in Josiah Macy Jr Found Confs on Blood Clotting and alli d probl ms J E Flynn ed 4 143 156 1951 Morphological and physiological studies of platelets and hemostasis

519 Zucker M B and J Borelli J. Appl. Physiol 7 425-431 1955 Quantity assay and release of serotonin in human platelets
520 --------J. Appl. Physiol 7 432-442 1955 Relationship of some blood clotting factors to serotonin release from washed platelets

SUPPLEMENTARY BIBLIOGRAPHY

521 Alexander B Fourth Internat. Congr. Biochem Symposium No 10 37-74 1958 Pergamon Press London etc Some biochemical physicochemical and immunochemical studies of prothrombin and proconvertin (Factor VII) their biopathologic significance
522 Biggs R and R B Macfarlane Human blood coagulation and its disorders 2nd ed C C Thomas Springfield 1957 476 pp
523 Didisheim F J H Ferguson and J H Lewis Arch. Int. Med 101: 347-354 1958 Hemostatic data in relatives of hemophiliacs A and B
524 Esnough M P and G V R Born Seventh Europ. Congr. Haematol Abstr 1959 196 The breakdown of phospholipids in plasma during clotting
525 Ferguson J H C L Johnston Jr and D A Howell Proc Soc. Exp. Biol. & Med Anti-AcG specific circulating inhibitor of the labile clotting factor
526 - -- - Blood 13 382-397 1958 A circulating inhibitor (anti-AcG) specific for the labile factor V of the blood clotting mechanism
527 Flynn J E and E T Standley in Josiah Macy Jr Found Confs on 'Blood clotting and allied problems' Flynn J E ed 2 145-160 1949 On the conversion of prothrombin to thrombin
528 Folch Pi J and F N Le Baron Fourth Internat. Congr. Biochem Symposium No 3 15 1958 Pergamon Press London etc Biochemistry of inositol lipides of the central nervous system
529 Fresh J W J H Ferguson and J H Lewis Obst. & Gyn 7 117-127 1956 Blood-clotting studies in parturient women and the newborn
530 Fresh J W J H Ferguson C Stamey F Morgan and J H Lewis Pediatrics 19 241-251 1957 Blood prothrombin proconvertin and proaccelerin in normal infancy questionable relationship to vitamin K
531 Fresh J W J H Lewis and J H Ferguson Tri State Med. J 14 18-20 1956 Hypocoagulability of the blood complicating obstetrics
532 Georgatsos J C C V Hussey and A J Quick Am. J. Physiol 181 30 34 1955 Nature and action of a new clotting factor obtained from erythrocytes

533 Hougie C E M Barrow and J B Graham J. Clin. Invest 36: 485 496 1957 Stuart clotting defect I Segregation of an hereditary hemorrhagic state from the heterogeneous group heretofore called stable factor (SPCA proconvertin factor VII) deficiency

534 Johnston C L Jr J H Ferguson and F A O'Hanlon Proc Soc. Exp. Biol. & Med 99 197-201 1958 Surface activation of plasma clotting A function of Hageman factor

535 - -- and W L Black Thromb. et Diath. Haem 3 367 374 1959 The fate of factor VII and Stuart factor during the clotting of normal blood

536 Johnston C L Jr J H Ferguson F A O'Hanlon and R B Payne Proc. Soc. Exp. Biol. & Med 101 747 750 1959 Isolation of coagulation factors by continuous flow electrophoresis

537 Johnston C L Jr and F A O'Hanlon Feder. Proc 18 77 (Abstr 303) 1959 Preparation of coagulation proteins by continuous flow electrophoresis

538 Lewis J H J H Burchenal R R Ellison J H Ferguson J H Palmer M L Murphy and M B Zucker Am. J Clin. Path 28: 433 446 1957 Studies of hemostatic mechanisms in leukemia and thrombocytopenia

539 Lewis J H J H Ferguson J W Fresh and M B Zucker J. Lab. & Clin. Med 49 211-232 1957 Primary hemorrhagic diseases

540 Lewis J H J H Ferguson E Spaugh J W Fresh and M B Zucker Blood 12: 84 89 1957 Acquired hypoprothrombinemia

541 Lewis J H D C Leary J W Fresh and J H Ferguson Obst. & Gyn 75 418-422 1958 Fibrinolytic fibrinogenopenia occurrence in a case of abruptio placentae

542 Lewis J H M B Zucker and J H Ferguson Blood 11 1073 1076 1956 Bleeding tendency in uremia

543 Ratnoff O D and J M Rosenblum Am. J. M.d 25 160 168 1958 Role of Hageman factor in the initiation of clotting by glass

544 Rodman N F E M Barrow and J B Graham Am. J. Clin Path 29: 525 538 1958 Diagnosis and control of the hemophilioid states with the partial thromboplastin time (PTT) test

545 Soulier J -P O Wartelle and D Menache Brit. J. Haematol. 5 121-138 1959 Hageman trait and PTA deficiency the role of contact of blood with glass

546 Tullis J L In Blood cells and plasma proteins, their state in nature J L Tullis ed Academic Press New York 1953 pp 143-155 The platelets of human blood their morphology interactions and preservation

547 Wagner P J and D A Farrell Thromb. et Diath. Haem 3 548 553 1959 Hydrolysis of tosylarginine methyl ester by Russell's viper venom

548 Wagner R H B A Richardson and K M Brinkhous Thromb et Diath. Haem 1 1-6 1957 A study of the separation of fibrinogen and anti hemophilic factor (AHF) in canine porcine and human plasmas

549 Wright I S Thromb. et Diath. Haem 3: 435 441 1959 Nomenclature of blood clotting factors

550 - -- -(chairman) and F Koller (editor) Montreux Conf (Aug 1959) of the International Committee for the Standardization of the Nomenclature of Blood Clotting Factors To be published in Thromb. et Diath. Haem